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## CHEMICAL CHANGES PRODUCED IN ISOTONIC SOLUTIONS OF SODIUM SULFATE AND SODIUM CHLORIDE BY THE SMALL INTESTINE OF THE DOG<sup>1</sup>

GLADYS R. BUCHER,<sup>2</sup> CARL E. ANDERSON<sup>3</sup> AND CHARLES S. ROBINSON

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ONE of the most baffling problems in physiology is the mechanism of the absorption of simple salts. Heidenhain (1), about 1884 concluded that absorption of even simple salines involved vital processes which worked along with, or in addition to, physical forces. Since his day a great many data have been collected in an attempt to evaluate the physical forces, but these, alone or combined, have been found inadequate to account for the transfers known to occur. The discovery that an enzyme assists in the absorption of glucose affords one example of how vital processes may participate since only living cells can produce enzymes. As yet no one has produced evidence that an enzyme is involved directly in the transfer of such ions as sodium and chloride, but Visscher and his co-workers (2-6) have accumulated evidence to indicate that the transfer of chloride by the lower small intestine of the dog cannot be explained by the laws governing simple diffusion or simple Donnan effect. Since the publication of their extensive data on the rates of transfer by the intestine of radio-sodium, radio-chloride and deuterium the whole subject has retreated into a meditative phase.

From this phase, however, some helpful ideas have emerged and the issues have been restated in the scientifically more acceptable terms of 'active transfer' and 'passive penetration.' Moreover, a set of criteria for active transfer has been formulated by Höber (7). Nevertheless, no one appears to have been able to evaluate the recorded data in terms of these criteria. The data on even the most studied of ions, i.e. chloride and sodium, as assessed by Höber himself and more recently by

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Ussing (8) are inadequate for the purpose of deciding that an active transfer is or is not involved.

Those who approach this problem through the literature will find the data already published inadequate for at least six reasons: 1) Much of the earlier data must be excluded because the intestinal loops had been rinsed with water which, because of its extreme hypotonicity, is injurious to the intestinal epithelium (9, 4). 2) Although it was known from the first that the upper segments of the gut behaved differently from the lower, the practice of using central segments has been prevalent. 3) Many investigators have been unaware that given loops show altered responses on consecutive tests and they have not avoided this source of variation. 4) Seldom have the chemical analyses been complete as regards total anion and total cation determinations. Thus the complete picture of the ion shifts and the influence of these shifts on membrane potentials can be delineated in but a few instances. The total base, carbon dioxide and pH have most often been ignored. 5) The great variability encountered from animal to animal makes it necessary to have a fair number of similar experiments so that statistical management of the data is feasible. Seldom has this been the case. 6) There has been relatively little attention given to the simple isotonic solutions in recent years. The most abundant data deal with half-isotonic solutions of sodium chloride and sodium sulfate, or with non-isotonic concentrations (10-12).

The aim of the present investigation was to furnish a complete background of data for a fresh start on the problems of ion transfer by the intestine. The specific objective was to determine the changes in volume, total cation and the various anions (sulfate, chloride, bicarbonate), ammonia and pH which occur when isotonic solutions of sodium sulfate or sodium chloride are introduced into upper and lower segments of the small intestine of the dog when three methods of isolating the intestinal segments are used: 1) the Miller-Abbott technique in anesthetized dogs, 2) the obstructive-clamp method in anesthetized dogs with intestinal loops exposed, and 3) the chronic dog prepared with a Thiry fistula loop of either the upper or lower small intestine.

#### EXPERIMENTAL PROCEDURE

The pattern of the experiments varied but little in the three methods of approach. In the acute experiments, fasted dogs (weighing 6 to 12 kg.) were anesthetized with sodium pentobarbital (Nembutal) (30 mg/kg.) and the intestine approached through a median abdominal incision. The upper segment (30-40 cm. long) was measured from the ligament of Treitz; the lower segment (25-40 cm. long) was measured from the ileo-cecal valve. The segments were opened by a small incision at the anti-mesenteric border and flushed clean with warm physiological saline. Just before each absorption test, the segments were rinsed once with an aliquot of the test solution. Then the test solution, usually 30 cc., was introduced and allowed to remain for the given time; the sulfate solutions for 20 minutes, the sodium chloride solutions for 10 minutes. The loop content was then collected by gravity and under mineral oil into a test tube for chemical analysis. The volume of the test solution remaining in the gut was estimated by the conductivity method originally described by Visscher *et al.* (11), using for the rinse 20 cc. of warm 10 per cent sucrose.<sup>4</sup> After an interval of nearly 10 minutes, the loops were again rinsed

<sup>4</sup> Conductivity measurements were made by an A. H. Thomas Ionometer at 25°C. and evaluated against conductivity obtained when known amounts of the tested specimen were added to a 20-cc. portion of sucrose solution.

with the test solution and subsequently filled for a repeat absorption performance. Each intestinal loop was used for but 2 test periods with the sulfate solution which was introduced at a concentration of 202 mEq/l.

In all experiments, the functional status of the loop was determined by its action on an aliquot of isotonic sodium chloride retained for 10 minutes. Usually this was done before, but sometimes both before and after, the two sulfate absorption periods. The sodium chloride solution was about 143 mEq/l.

The Miller-Abbott technique was used in 6 experiments on 6 dogs. The terminal portion of the triple-lumen tube with proximal and distal balloons was introduced through the small incision used in flushing the segment clean. One Miller-Abbott tube was inserted caudally from the ligament of Treitz and another orally from the ileo-cecal valve. The tubes were secured at the site of the incision by a purse-string ligature in the gut wall. The free ends extended outside the abdominal cavity which was closed as soon as possible. The interballoon spaces (25-35 cm. long) were isolated by inflating the balloons with air to a pressure of 15 to 20 mm. Hg, which simulates the maximal physiological pressure (13). This pressure was maintained throughout the absorption period. Following the sucrose rinse, the balloons were deflated, and later re-inflated for the next test.

In four other dogs, the upper and lower intestine was brought outside the abdominal cavity and arranged on saline-moistened sponge-rubber electric heating pads. An intestinal catheter with a small 'dead volume' was tied into the opened end of the intestine and the other end of the loop was closed by a flat clamp made by fitting two solid glass rods about 2 inches long into a rubber tube 4 inches long until the rods were about 0.5 inch apart at the center. This device, when inserted between the vessels at the mesenteric border and the ends held together with a rubber band at the anti-mesenteric border provided a satisfactory clamp which did not distort the local circulation as a tape ligature would. The intestines were covered with other heating units so the temperature was held at 37 to 40°C. In *experiment 12*, the temperature accidentally rose to nearly 45°C. in the lower loop and this may account for the marked changes this loop produced on its content.

The chronic animals were one to 4 weeks post-operative. When the loops were not exercised in experimentation, they were rinsed almost daily with isotonic glucose or saline to maintain them in good condition. The experiments were conducted on the animals while standing in a Pavlov stock. A special multi-perforate catheter was used. Leakage was prevented by the use of two balloons, one of which was inflated internally and one externally to the stoma, as described by Johnston (14). The pressure in the internal balloon was 15 to 20 mm. Hg.

All samples were analyzed immediately for pH and total carbon dioxide. The pH was determined with a Cambridge electron-ray pH meter at 25 to 28°C. The total carbon dioxide was determined manometrically by the method of Van Slyke and Neill (15). The total base and sulfate were determined by a modification of the benzidine method. Chloride was measured by direct titration with standard silver nitrate using potassium chromate as the indicator, and ammonia was determined by the micro-method of Conway and Byrne (16). All the chemical determinations, including controls on known standards, were made in duplicate.

TABLE 1. RESULT OF CHEMICAL EXAMINATION OF ISOTONIC SULFATE RETAINED FOR TWO 20-MINUTE PERIODS IN JEJUNAL LOOPS OF DOGS. FIRST-PERIOD DATA IN COLUMN 1, SECOND-PERIOD DATA IN COLUMN 2

DOG	VOLUME PUT IN		VOLUME OUT (% OF VOL. PUT IN)		TEST SOLUTION SUL- FATE PUT IN		PH OF SOL. RECOVERED (T = 20°C.)		ANALYSIS OF TEST SOLUTION RECOVERED, CONCENTRATION mEq/l.										TOTAL IONS <sup>3</sup>			
									TOTAL BASE		(NH <sub>4</sub> )		SULFATE		CHLORIDE		CO <sub>3</sub>					
	1	2	1	2	1 <sup>1</sup>	2 <sup>2</sup>	1	2	1	2	1	2	1	2	1	2	1	2				
	ml.				mEq/l.																	
3	30	30	46.6 <sup>4</sup>	51.6 <sup>4</sup>	204.5	203.0	6.29	6.28	218.8	214.5	2.9	2.1	163.0	177.1	39.8	24.3	2.8	1.6	221.7 c	215.8		
																			205.4 a	208.6		
4	30	30	103.0	95.6	202.4	201.7	6.04	5.42	215.3	213.2	2.1	1.8	180.5	175.1	32.7	27.4	1.4	1.0	217.4 c	215.0		
																			215.4 a	204.4		
5	30	30	96.7	112.7	204.4	197.6	6.06	6.01	218.9	213.8	2.8	2.6	179.1	176.5	40.0	35.8	2.0	2.1	221.7 c	216.4		
																			221.1 a	214.3		
6	30	30	103.6	108.0	204.7	199.4	6.05	6.00	212.0	212.0	2.5	1.3	183.2	191.2	31.3	26.8	1.2	0.8	214.5 c	213.3		
																			215.5 a	218.7		
7	30	30	130.3	111.7	202.7	202.7	6.79	6.49	203.8	206.3	—	—	154.0	170.1	47.4	33.8	2.9	1.1	205.6 c	215.2		
																			204.3 a	205.0		
8	30	30	105.0	113.3	197.3	199.8	6.00	5.84	210.6	210.6	2.9	2.4	157.6	176.5	44.8	30.5	1.3	1.5	213.5 c	213.0		
																			203.7 a	208.5		
Mean, Miller-Abbott seg-ments																						
			107.7	108.2			6.12	6.02	213.2	211.7	2.6	2.0	169.5	177.7	39.3	29.8	1.9	1.3	215.7 c	214.8		
																			210.9 a	210.0		
9	30	30	115.6	115.3	204.0	200.0	6.36	6.43	213.0	220.2	0.0	2.4	177.1	180.6	36.7	30.7	2.2	2.4	213.0 c	222.6		
																			216.0 a	213.1		
10	30	30	115.0	111.3	205.4	204.8	6.26	6.26	222.7	220.6	3.9	4.3	178.2	181.3	34.7	32.1	2.3	2.5	226.6 c	220.6		
																			215.2 a	215.9		
11	30	30	107.0	101.3	200.2	199.8	6.18	6.16	216.7	211.3	2.7	2.1	192.7	190.1	30.3	30.9	1.2	1.9	219.4 c	211.3		
																			224.2 a	222.9		
12	30	30	107.3	98.3	206.2	200.7	6.28	6.11	213.3	206.1	3.3	1.8	180.7	181.2	33.1	28.8	2.5	2.5	216.6 c	206.1		
																			216.3 a	212.5		
Mean, ligated loops																						
			111.2	104.3			6.27	6.24	216.4	214.5	2.5	2.6	182.2	183.3	33.7	30.6	2.0	2.3	218.9 c	215.1		
																			217.9 a	216.1		

<i>Br</i>	40	40	120.0	119.5	201.6	199.8	6.49	6.40	211.3	212.0	2.1	1.9	173.6	181.9	36.1	30.3	2.6	2.8	213.4 c 213.9
<i>Br</i>	35	35	119.4	114.0	199.8	200.5	6.04	6.06	221.4	226.1	2.1	1.0	183.0	190.1	37.9	33.7	0.8	0.8	212.4 a 215.0
<i>Br</i>	35	35	119.7	110.6	205.9	202.3	6.06	6.07	217.1	217.1	2.2	1.8	177.0	195.2	39.9	25.8	1.7	1.8	223.5 c 227.1
<i>Bl</i>	28	28	111.4	109.3	206.4	202.3	6.42	6.22	218.5	219.6	2.2	1.8	176.9	185.8	42.2	40.8	2.5	2.0	221.7 a 224.6
<i>Bl</i>	25	25	114.8	111.2	201.4	204.1	6.15	6.18	216.0	216.0	2.5	1.5	173.5	173.5	42.6	43.4	1.2	1.6	219.3 c 218.9
<i>Bl</i>	29.5	25	104.0	111.6	201.4	201.6	6.47	6.18	210.2	216.0	2.9	2.3	161.5	173.5	47.7	43.4	2.7	1.7	218.6 a 222.9
Mean, Thirty-Villa loops																			
			114.9	112.7			6.27	6.18	215.7	217.8	2.3	1.7	174.2	183.3	41.1	36.2	1.9	1.8	220.7 c 221.4
																			211.6 a 228.6
																			218.5 c 217.5
																			217.3 a 218.3
																			213.1 c 218.3
																			211.9 a 218.6

5

Mean of 16	111.5	109.6					6.24	6.13	215.0	214.7	2.5	2.1	174.5	181.2	38.6	32.4	2.0	1.8	217.4 c 216.6
Diff. of means with S.E.	1.9 ±	2.1					0.108 ± 0.04 <sup>5</sup>	0.3 ±	0.3 ±	1.0	0.4 ± 0.23	6.7 ±	1.9 <sup>5</sup>	6.2 ±	1.3 <sup>5</sup>	0.2 ±	0.16		215.0 a 215.7

<sup>1</sup> As determined by sulfate analysis.<sup>2</sup> As determined by total base analysis.<sup>3</sup> Cations = c; anions = a.<sup>4</sup> Omitted in the average.<sup>5</sup> Significant.

## DATA AND DISCUSSION

The data have been arranged into three tables: table 1 contains all data on isotonic sodium sulfate retained for 20 minutes in 2 consecutive periods in jejunal loops, table 2 contains parallel data on the lower or ileal loops and table 3 contains the data for the tests using isotonic sodium chloride retained for 10 minutes. The mean values of all measurements for each of the 3 types of animal preparation have been inserted in all the tables. Since, however, these mean values varied to about the same degree as the values within a group, no one of the three types of animal preparation could be singled out as decidedly different, or superior, and this finding was made the justification for combining the data from all experiments as given by the means at the bottom of the full tables.

The functional characteristics of the upper and lower gut which are evident when it is acting on isotonic sodium chloride will be discussed first, with repeated reference to the data in table 3. Sodium chloride (143 mEq/l.) was readily absorbed from the upper and lower segments, but especially from the latter, where in 10 minutes 18 per cent (an average of 5.4 cc.) was absorbed. The next item of interest is the marked increase in the pH of the lower-loop content and its high carbon dioxide value as compared with that of the upper loops (see *columns F and J*). These data are similar to those on the human obtained by Bucher, Flynn and Robinson (17) and bear out their contention that the change in pH is secondary to an anion exchange in which bicarbonate ion enters the gut lumen while chloride ion leaves; the total cation and anion concentration remaining unchanged. In the present study, the total cation and total base concentrations in the withdrawn content were 145 and 143 mEq/l. respectively and the total base of the saline as introduced was 143 mEq/l. Likewise, the total anion concentration in the withdrawn content was 143 mEq/l. Thus, while the chloride was removed, by an average of 15 mEq/l., there entered 12.3 mEq/l. of bicarbonate ion, as calculated from the total carbon dioxide value and the pH by the use of the Henderson-Hasselbalch equations (18). If other anions were present, their concentrations were extremely low and within the range of error of the methods used. The indications are that an equivalent exchange of chloride for bicarbonate occurred.

These data illustrate how estimates of *sodium* chloride uptake from the gut based on *only the chloride* determinations can be misleading since chloride is partially taken up without its equivalent of base.

The mechanism which underlies this exchange and its accentuation in the ileum is not clear. The direction of bicarbonate movement and pH suggests that in this region the intestinal content is advancing toward equilibrium with the blood and tissue fluid. If this be so, what prevents or inhibits this diffusion in the upper segment? We have no answer yet. Bicarbonate has long been regarded as a normal secretory product of the intestine, but the observation that it enters the intestine at the same rate at which chloride leaves suggests that it is not simply secreted but is a part of a more complicated process, involving chloride absorption.

The data reveal another interesting difference between upper and lower segments. When sodium chloride, 143 mEq/l., was introduced into the jejunal loops, it *gained* in total base, so that after 10 minutes it was 148 to 152 mEq/l. When the

same solution was introduced into the ileal loops, there was *no change* whatever in the total base concentration. Visscher and Roepke (12) have observed this same feature from a different direction. They found that sodium chloride solution (153–160 mEq/l.) became mildly hypotonic while undergoing absorption in ileal loops to the extent of about 6 mM NaCl per kg. of water. Taken as a whole, the evidence indicates that the ileal mucosa has the ability to render its content hypotonic, (relative to plasma, 158 mEq/l.) to an equilibrium level of approximately 143 mEq/l., but if this concentration is present as introduced, the solution is absorbed without adjustments in its total base or tonicity. This imposes certain limits on the Fluid Circuit Theory of intestinal absorption.

In four instances the intestinal loops were tested before and also after the sulfate test periods with sodium chloride solution. The data (see table 3, *dogs 9–12*) show that the ability of the intestine to produce the anion exchange is not affected by a previous exposure to isotonic sulfate, or vice versa. If sulfate ion alters the transfer mechanisms of the intestinal epithelium, this alteration is present only as long as the sulfate ion is present. The regular behavior of the loops with respect to sodium chloride may dispel any doubts that the intestinal loops were non-functional or abnormal with respect to the sulfate solution.

Turning now to the sulfate studies, where a double absorption study was made on each loop, it was first necessary to determine if the results were reliable, in the sense of being duplicates. Before comparing upper- and lower-loop data, it was first necessary to compare first- and second-period data at each level. Accordingly all data on the first 20-minute samples were paired with corresponding data from the second-period samples from a given loop and the several differences subjected to statistical analysis. By the customary rule, if a mean difference equals, or exceeds twice its standard error, the probability is less than 5 in 100 that such a difference would occur by chance and the difference is regarded as having significance.

In the upper loops, differences due to factors other than chance were discovered in three of the measurements. The pH and chloride concentration were higher and the sulfate concentration lower in the content remaining after the first absorption period than in that after the second. In all other measurements, i.e. volume recoveries, total base, ammonia,<sup>6</sup> carbon dioxide, total anion and total cation, the values for the two periods were the same.

Upon similar analysis of the data from the lower loops, the pH of the first specimen was again significantly higher, but the only other item in which a significant difference was found was the total base, which was higher in the first-period specimens.

One conspicuous feature of the upper loops was a definite fluid contribution, amounting to 7 to 15 per cent, which did not occur in the lower loops. Could any of the differences here be related to the extent of the fluid contribution? This point was examined in the following manner. The pairs of data for the two absorption tests were sorted into two groups on the basis of volume recovered in the second period.

<sup>6</sup> In both the saline series and the sulfate experiments, the magnitude of the concentration changes observed seemed unrelated to the ammonia content. This observation confirms the doubt expressed by Ingraham and Visscher (5) and re-emphasized by Ussing (8) that ammonia plays any role in the sodium and chloride ion transport from gut to blood.



TABLE 2. RESULTS OF CHEMICAL EXAMINATION OF ISOTONIC SULFATE RETAINED FOR TWO 20-MINUTE PERIODS IN ILEAL LOOPS OF DOGS. FIRST-PERIOD DATA IN COLUMN 1, SECOND-PERIOD DATA IN COLUMN 2

ANALYSIS OF TEST SOLUTION RECOVERED, mEq/l.																					
DOG	VOLUME OUT (% OF VOL. PUT IN)				TEST SOLUTION PUT IN		ME OF SOL. RECOVERED ( $T = 20^{\circ}\text{C}.$ )		ANALYSIS OF TEST SOLUTION RECOVERED, mEq/l.										TOTAL IONS <sup>a</sup>		
	VOLUME PUT IN		VOLUME OUT		SUL- FATE	TOTAL BASE	TOTAL BASE		(NH <sub>4</sub> )		SULFATE		CHLORIDE		CO <sub>3</sub>						
	1	2	1	2			1	2	1	2	1	2	1	2	1	2	1	2			
mEq/l.																					
3	30	85.0	72.3	204.5	203.0	6.01	6.10	218.8	218.8	1.0	0.2	218.4	213.4	0.2	0.2	2.2	1.5	227.0	221.1	221.0	214.9
4	30	96.7	126.0	202.4	201.7	7.39	7.21	200.9	200.9	2.2	1.7	161.2	161.2	15.8	19.7	16.4	19.0	207.8	202.6	207.8	202.6
5	30	98.7	97.0	204.4	197.6	6.75	6.94	219.2	213.8	1.5	0.3	219.7	198.4	1.0	3.5	4.2	7.6	193.9	201.0	193.9	201.0
6	30	85.7	85.0	204.7	199.4	6.25	6.10	222.4	219.6	3.6	3.5	219.4	219.4	0.7	0.7	2.6	1.6	226.0	223.1	224.9	209.3
7	30	88.7	85.0	202.7	202.7	7.23	6.93	216.7	218.2	1.6	2.2	197.3	206.3	7.3	3.1	9.2	4.7	218.3	220.4	214.1	214.1
8	30	124.7	132.7	197.3	199.8	7.32	7.25	201.6	199.8	2.6	3.8	148.8	163.7	23.4	16.9	25.7	18.7	204.2	203.6	197.9	199.2
Mean, Miller-Abbott segments.																					
	96.6	99.6	6.82	6.75	215.2	211.8	2.1	1.9	194.1	193.7	8.0	7.3	10.0	8.8	217.3	214.1	212.3	210.0			
9	30	101.3	98.3	204.0	200.0	6.97	6.77	220.9	216.4	1.8	0.0	209.8	206.3	0.2	0.3	5.1	5.2	222.7	216.4	215.1	211.8
10	30	93.3	82.7	205.4	204.8	6.51	6.51	230.3	226.4			220.5	219.1	3.1	0.2	4.8	4.6	234.6	226.4	228.4	223.9
11	30	96.7	90.3	200.2	199.8	6.68	6.29	218.2	213.5			210.5	218.7	0.2	0.1	3.1	2.3	220.3	213.5	213.8	221.1
12	30	118.0	138.3	206.2	200.7	7.45	7.42	215.5	198.6			160.5	149.3	21.4	22.4	24.9	28.0	217.3	198.6	206.8	199.8
Mean, ligated loops																					
	100.8	102.4	6.90	6.75	221.2	213.7	200.3	198.3	6.2	5.7	9.5	10.0	223.7	213.7	216.0	214.1					

<i>Sh</i> 30	28	85.3	120.7	199.1	201.1	6.93	6.83	238.3	230.8	2.2	1.6	223.2	224.6	5.6	4.4	5.6	4.9	240.5 c	232.4
<i>Sh</i> 30	30	92.3	92.3	204.1	201.4	7.17	6.82	221.0	220.0	2.2	2.1	206.2	213.3	7.9	3.4	8.7	5.9	235.4 a	233.9
<i>Sh</i> 30	30	96.3	93.7	201.6	201.4	7.12	7.08	229.3	228.6	2.1	1.3	208.5	206.5	8.5	10.4	8.6	9.4	222.2 c	222.6
<i>Te</i> 30	30	96.3	102.3	195.5	201.4	6.88	6.60	224.3	224.3	2.3	1.7	210.5	210.0	6.2	0.2	5.2	3.9	231.4 c	229.9
<i>Te</i> 25	25	100.8	100.8	201.6	201.4	7.02	6.90	220.7	225.7	1.6	1.5	207.2	213.1	7.0	4.5	7.7	6.5	225.6 a	226.3
<i>Te</i> 25	25	87.6	90.4	199.1	203.4	6.95	6.86	225.4	229.3	1.0	2.3	213.9	225.8	4.5	1.4	6.7	3.2	221.9 a	223.1
<b>Mean, Thirty-Villa loops</b>																			
		93.1	100.0			7.01	6.85	226.5	226.4	1.9	1.7	211.6	217.0	6.6	4.0	7.2	5.6	228.2 c	228.8
																		226.5 a	226.5
Mean of 16.	93.3	100.5				6.91	6.79	221.0	217.8	2.0	1.7	202.3	203.6	7.0	5.7	8.9	7.9	223.0 c	219.3
Differences of means with S.E.	4.2 ±	3.3				0.12 ± 0.04 <sup>1</sup>		2.9 ±	1.0 <sup>4</sup>	0.3 ± 0.24		1.4 ±	2.3	1.3 ± 0.76	0.91 ± 1.3			218.5 a	216.8

<sup>1</sup> As determined by sulfate analysis.<sup>2</sup> As determined by total base analysis.<sup>3</sup> Cations = c; anions = a.<sup>4</sup> Significant.

*Group A* contained all pairs of data where the smaller volume was recovered in the second sample, and *group B* contained those pairs of data where the larger volume was recovered in the second period. Except for the volume, which was the basis for the group distinctions, no difference appeared to correlate with the differences in volume recoveries. Therefore, the cause for the finding of differences in the second-period specimen as compared with the first-period specimen cannot be associated with the difference in volume recovered, and by this analysis would appear to be *independent of the fluid secretion* which occurred in the upper segment. By similar reasoning applied to the data of the lower loops, the cause for the observed differences in the specimen from the two periods would appear to be *independent of the fluid absorption* which occurred in these loops. No explanation that is satisfactory can be given at the present time for the differences observed in the two consecutive absorption specimens. The findings make it inadvisable to group or pool data from successive absorption tests on the same intestinal loop. They also demonstrate another way in which the physiology of upper-loop absorption of salines differs from that of lower loops.

Previous investigators seem to have overlooked the pH gradient which is illustrated in the present data on isotonic sulfate solutions. Compared to the gradient observed when isotonic sodium chloride was present in the gut, the pH is adjusted half a pH unit to the acid direction in the presence of the sulfate. Thus the sulfate solution was significantly less alkaline at all levels and in the lower ileum seldom attained a value greater than pH 7.0.

The low diffusibility of the sulfate ion impels one to consider the Donnan effect of this substance in the gut. There is a suggestive resemblance in the present data to the ion distribution observed by Netter and designated by Höber as type 2 Donnan. Netter (19) separated a solution of M/1400 potassium sulfate from a mixture of M/1400 potassium sulfate + M/10 sulfuric acid by means of a dried collodion membrane. The two compartments were kept in osmotic balance by the addition of glucose solution to the side containing only the potassium sulfate. After two weeks, he discovered that potassium had migrated against the gradient, and increased to a concentration 10 times its initial value in the compartment containing the sulfate and the sulfuric acid. The experiment should be made where the osmotic balance on the one side is achieved through the use of plasma while isotonic sodium sulfate is on the other. When such data becomes available, perhaps the present data along with a better knowledge of the gradient of electro-negative potential which exists in the small intestine, will make possible a more exact evaluation of the participation of active and passive transfer mechanisms here.

Of more interest now, is the reinforcement the present data lends to a point brought out by Visscher and Roepke (12) regarding chloride entrance into sulfate solutions being absorbed in ileal loops. They state in summary, "The net increase in chloride concentration in sulfate solutions was very small, on the average, 4 milliequivalents per liter in 40 minutes. Consideration of isotopic tracer data leads one to believe that this represents a small fraction of the chloride that entered in that time. The remainder must have returned to the blood by some active transport mechanism." In the present study, the total base of the sulfate solution increased

TABLE 3. SUMMARY OF CHEMICAL CHANGES IN ISOTONIC SALINE PRODUCED IN 10 MINUTES BY UPPER AND LOWER INTESTINAL LOOPS IN DOGS

GROUP <sup>1</sup>	DOG	A	B	C	D	E	F	G	H	I	J	K	L
		VOL. IN cc.	VOL. OUT cc.	VOL. OUT %	TEST SOLUTION		VALUES ON SOLUTION RECOVERED						
					mEq./l.		mEq./l.						
					TOTAL BASE	CHLO- RIDE	pH	TOTAL BASE	NH <sub>3</sub>	CHLO- RIDE	CO <sub>2</sub> <sup>2</sup>	TOTAL CATION	TOTAL ANION
<i>A. Upper Loops</i>													
<i>G</i> <sub>1</sub>	5	30 <sup>3</sup>	30.7 <sup>3</sup>	102.3	140.0	143.5	6.11	151.2	1.3	141.7	1.7	152.5	155.1
	6	30	29.9	99.6	141.4	142.1	6.08	148.3	3.3	152.5	0.9	151.6	153.4
	7	30	31.8	106.0	141.1	143.3	6.72	142.6	1.8	147.2	1.6	148.0	148.8
	8	30	23.6	78.6	144.0	143.8	6.08	150.1	2.3	149.5	1.2	152.4	150.7
Mean of 4				96.6	141.6	143.3	6.25	148.0	2.2	147.7	1.3	151.1	152.0
<i>G</i> <sub>2</sub>	9	30	24.0	80.0	142.4	142.6	6.47	145.0	2.2	148.1	1.9	147.2	150.0
	9	30 <sup>3</sup>	24.7 <sup>3</sup>	82.5	142.6	142.4	6.45	155.8		149.5	1.9	155.8	151.4
	10	30	27.1	90.3	142.5	142.6	6.46	148.6	3.6	151.4	2.1	152.2	153.5
	10	30 <sup>3</sup>	25.0 <sup>3</sup>	83.3	142.5	142.0	6.32	151.1		145.5	2.0	151.1	147.5
	11	30	23.6	78.7	143.3	143.0	6.30	144.7		146.1	1.0	144.7	147.1
	11	30 <sup>3</sup>	24.0 <sup>3</sup>	80.0	143.3	143.0	6.27	146.9	1.9	143.1	1.5	148.8	144.6
	12	30	23.9	79.7	143.4	143.4	6.78	150.3		148.1	2.3	150.3	150.4
	12	30 <sup>3</sup>	22.5 <sup>3</sup>	75.0	143.4	143.4	6.60	148.9	2.4	144.4	1.9	151.3	146.3
	Before means			82.2	142.9	142.9	6.50	147.1		148.4	1.8	148.8	150.2
	After means			80.2	142.9	142.9	6.43	150.6		145.6	1.8	151.7	147.7
<i>G</i> <sub>2</sub>	<i>Br</i>	40	40.6	101.5	140.0	142.1	6.44	141.5	2.0	143.8	2.5	143.5	146.3
	<i>Br</i>	40	43.6	109.0	145.4	140.9	5.92	152.6	1.6	154.6	1.2	154.2	155.2
	<i>Br</i>	35	38.2	109.1	140.4	141.0	6.14	145.1	2.0	146.1	1.7	147.1	147.8
	<i>Bl</i>	29.5	30.8	104.4	139.3	142.7	6.54	148.3	1.9	152.5	2.7	150.2	155.2
	<i>Bl</i>	25	27.0	108.0	140.8	142.7	6.30	148.3	1.8	149.7	1.5	150.1	151.2
	<i>Bl</i>	25	25.3	101.2	140.0	142.9	6.46	149.8	2.9	148.3	1.8	152.7	150.1
Means of 6				105.5	141.6	142.0	6.30	149.1	2.0	146.1	1.6	149.3	150.9
Means of all 18				92.7	142.2	142.7	6.36	148.3	2.2	147.9	1.7	150.2	150.2
<i>B. Lower Loops</i>													
<i>G</i> <sub>1</sub>	5	30 <sup>3</sup>	20.3 <sup>3</sup>	67.7	140.0	143.5	7.08	154.8	0.3	112.0	10.6	155.1	150.0
	6	30	23.7	79.0	141.4	142.1	7.23	142.5	11.1	135.9	10.5	153.6	146.4
	7	30	21.8	70.3	141.1	143.3	7.53	142.0	2.3	133.1	12.5	145.2	145.6
	8	30	24.4	81.0	144.0	143.8	7.11	141.8	3.0	136.2	10.0	144.8	146.2
Mean of 4				74.5	141.6	143.2	7.24	145.5	4.1	129.3	10.9	149.8	147.0
<i>G</i> <sub>2</sub>	9	30	23.7	79.0	142.4	142.6	7.56	146.1	2.6	133.6	12.2	148.7	145.8
	9	30 <sup>3</sup>	21.6 <sup>3</sup>	105.3	142.4	142.6	7.19	147.9		130.8	9.0	147.9	148.6
	10	30	24.8	80.3	142.5	142.6	7.11	152.2	4.0	135.1	11.0	156.2	146.1
	10	30 <sup>3</sup>	20.1 <sup>3</sup>	67.0	142.5	142.6	7.15	150.4		130.9	14.4	150.4	145.3
	11	30	20.1	67.0	143.3	143.0	7.24	139.3		133.5	7.1	139.3	140.6
	11	30 <sup>3</sup>	22.1 <sup>3</sup>	73.6	143.3	143.0	7.15	137.9	1.7	131.8	6.9	139.6	138.7
	12	30	34.0	113.3	143.4	143.4	7.60	142.0		125.1	21.5	142.0	146.6
	12	30 <sup>3</sup>	41.8 <sup>3</sup>	139.3	143.4	143.4	7.75	143.5	1.2	115.3	29.7	144.7	145.2
	Before means			84.9	143.9	143.9	7.40	144.9		131.8	12.9	146.5	144.8
	After means			96.3	143.9	143.9	7.37	144.9		127.2	17.7	145.6	144.4
<i>G</i> <sub>2</sub>	<i>Sh</i>	30	23.5	78.3	140.4	142.7	7.60	144.0	1.0	132.0	17.3	147.2	140.3
	<i>Sh</i>	30	17.8	59.3	142.9	146.2	7.40	137.2	2.1	118.7	18.5	139.3	137.2
	<i>Sh</i>	30	17.3	57.6	140.8	142.9	7.55	142.6	1.7	123.0	17.6	144.3	140.6
	<i>Ta</i>	30	24.6	82.0	140.4	142.7	7.05	125.3	1.2	122.7	6.0	120.5	128.7
	<i>Ta</i>	25	23.0	92.0	144.0	143.1	7.35	135.7	1.7	124.1	10.8	137.4	134.9
	<i>Ta</i>	25	19.0	76.0	142.9	140.8	7.21	148.3	1.0	133.4	11.7	140.3	145.1
Mean of 6				73.6	141.9	143.1	7.33	138.8	1.4	125.6	13.3	140.6	139.6
Mean of all 18				81.5	142.8	143.0	7.34	143.0	2.5	128.0	13.2	145.1	143.3

<sup>1</sup> *G*<sub>1</sub> = Miller-Abbott segments. *G*<sub>2</sub> = Clamped segments. *G*<sub>2</sub> = Chronic loops. <sup>2</sup> Values are for total CO<sub>2</sub> in mm/l. Since most of this is present as univalent bicarbonate ion one mm is approximately equal to one mEq. <sup>3</sup> Absorption period after the 2 sulfate-absorption periods.

16 to 20 mEq/l. If the base entered the gut attended by its equivalent of chloride, when but 7 mEq/l. were found, at least 9 mEq/l. must have returned to the blood, exchanging for bicarbonate. In the lower segments nearly 9 mEq/l. of bicarbonate was the finding. The requirements of Visscher and Roepke's surmise and our data agree, and we may speculate that the active transport mechanism ties in with the exchange of bicarbonate ion for chloride ion.

#### SUMMARY

Data are presented which show the changes in volume, total cation, sulfate, chloride, bicarbonate, ammonia and  $H^+$  ion concentrations which occur when an isotonic solution of sodium sulfate (202 mEq/l.) was introduced into jejunal and ileal segments of 12 dogs in two successive 20-minute periods. The same items of data are presented which show the changes when sodium chloride (143 mEq/l.) was introduced into jejunal and ileal loops of 12 dogs in a single 10-minute period.

The data accruing from three types of animal preparation, 1) the Miller-Abbott technique, 2) the exposed clamped segment and 3) the Thiry fistula, are sufficiently homogenous to indicate that no one of these techniques can be singled out as the better.

When sodium chloride (143 mEq/l.) was introduced into jejunal loops, it gained in its concentration of total base. When the same solution was introduced into ileal segments, the total base remained the same as introduced, although 20 per cent of the solution was absorbed.

When sodium chloride (143 mEq/l.) was introduced into jejunal loops, the mean pH was 6.36. When sodium sulfate (202 mEq/l.) was introduced into jejunal loops the mean pH was 6.24 in the first period. In the ileal loops, the values of pH obtained, when these two solutions were introduced, were 7.54 and 6.91, respectively.

Two consecutive absorption tests on a given loop yield data which cannot be considered as duplicate tests on the basis of the chemical findings. In this regard, upper and lower loops are similar, but they differed in respect to the several ions, as can be examined in the statements below.

In jejunal loops, the second-period findings differed significantly from those of the first in possessing a greater sulfate concentration (181 vs. 174 mEq/l.) and lower pH and chloride concentrations (pH 6.13 vs. 6.24 and chloride of 32 vs. 38 mEq/l.).

In the ileal loops the second-period findings differed significantly from those of the first in having a lowered total base (218 vs. 221 mEq/l.) and a lower pH (pH 6.79 vs. 6.91).

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## COOLING AS A STIMULUS TO SMOOTH MUSCLES<sup>1, 2</sup>

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COOLING may cause smooth muscles to contract, as indicated by studies on the oesophagus of the toad (1), the retractor penis (2) and the intestine (3, 4) of the dog, the iris of the cat under certain conditions (5) and the dartos and cremaster muscles of the ram (6) in which the response appears to play an important role in maintaining the testis at a temperature suitable for spermatogenesis. In these various experiments, rewarming produced a diminution of tone and lengthening of the muscle except in the case of the iris of the cat which contracted on warming from 2°C., reaching a maximum height at 30°C. (5).

On the other hand, cooling sometimes causes smooth muscles to lengthen, as reported for the iris of the cat with extreme cooling (5, 7), for the bladder of the dog (8) and for isolated arteries, using longitudinal preparations (9) or perfused segments (10), though local cooling of the intact digital arteries of man caused them to constrict, apparently by a direct action on the vessel (11).

Starling (12) and Evans (13) emphasize the fact that rapid cooling is a more effective stimulus than gradual cooling, as evidenced by the fact, noted by Starling, that rapid cooling of the retractor penis muscle of a dog from 35° to 25°C. causes as large a contraction as slow cooling to 5°C.

Our interest in cooling as a stimulus to smooth muscles was aroused when we observed that sudden vasoconstriction occurred in sympathectomized or denervated paws exposed to cold, presumably because of a direct effect of cold on the arterio-venous anastomoses of the skin, or the arteries supplying them (14). It was therefore decided to study the effect of cooling the nictitating membrane of the cat, a smooth muscle placed by Bozler (15) in the same classification, i.e. 'multi-unit,' as the arterioles, and, unlike the gut, probably free of ganglion cells whose behavior might complicate the response (16).

This paper reports the results of cooling the nictitating membranes of the cat *in situ*, and, subsequently, of cooling the retractor penis of the dog and other smooth muscles, in a muscle bath. The results indicate: 1) that cooling acts as a stimulus to contraction in all smooth muscles so far studied, the response varying with the degree and rate of cooling as stated by Starling; 2) in some experiments, the chroni-

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cally denervated nictitating membrane becomes 'sensitized' to cooling as well as to epinephrine; 3) cooling apparently acts on a different system than epinephrine, as indicated, first, by a comparison of the response curves for cooling on the one hand, and epinephrine on the other; second, by studies using the adrenolytic drug Di-benamine,<sup>4</sup> which abolishes the excitatory effect of epinephrine but not of cooling; and third, by studies involving the algebraically additive effects of cooling and epinephrine when administered simultaneously.

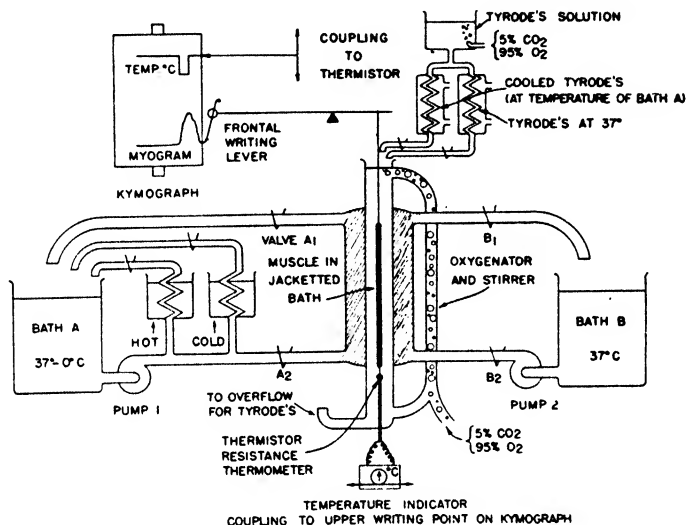


Fig. 1. APPARATUS USED FOR RAPID OR GRADUAL COOLING of isolated smooth muscles, with simultaneous recording of muscular contractions and temperatures. For cooling the nictitating membrane of the cat, glass bulbs were placed in the orbits following enucleation of the eyes. These bulbs were inserted in the system in place of water jacket of the muscle bath.

#### METHODS

*In situ Cooling of Nictitating Membranes, Cat.* The animal was anaesthetized with sodium pentobarbital (veterinary Nembutal) 0.5 ml/kg. *In situ* cooling of the nictitating membranes was effected by means of hollow glass bulbs, the same size as the eye, which were placed in the orbits following careful enucleation of the eyes. Water was pumped through the interior of the glass bulbs and out past a thermometer which provided the only (rough) measure of the temperature of the nictitating membrane. The water, initially at 37°C., could be rapidly or gradually cooled by means of the apparatus described below.

The cat was placed on its back on a board elevated above the table. Threads were led directly upward, without pulleys, from each nictitating membrane to isotonic heart levers with 'fall-away' writing points, arranged, one above the other, so that a contraction was recorded as an upward excursion.

<sup>4</sup> Kindly furnished by Dr. William Gump of the Givaudan-Delawanna Laboratories, Delawanna, N. J.



Contractions of the nictitating membranes as a result of activity of the autonomic nervous system were prevented by sectioning the cervical sympathetic trunks and, in some animals, by acute adrenalectomy on the day of experiment. Motions of the nictitating membranes resulting from movements of the eye muscles were eliminated by full curarization with 'intocostrin,' the adequacy of dosage being tested by continuous stimulation, at one per second, of one sciatic nerve. In the curarized animals, artificial respiration was maintained via tracheal cannula.

For studies on the chronically denervated nictitating membrane, one superior cervical ganglion was removed aseptically a week prior to the experiment.

*Retractor Penis, or other Smooth Muscles—Muscle Bath.* Each muscle was carefully dissected from an anesthetized animal (frequently just used for another experiment), and was placed in Tyrode's solution in a glass bath with water jacket. A 5 per cent carbon dioxide, 95 per cent oxygen mixture was bubbled through the solution, the pH of which was kept at 7.4 by maintaining the  $p\text{CO}_2$  at 36 mm. and the bicarbonate ion at 20 mM/l., as calculated from the Henderson-Hasselbalch equation. Isotonicity was maintained by keeping the sum of the sodium chloride and sodium bicarbonate at 147 mM/l. in a Tyrode's solution otherwise identical in composition to that described by Carrel (17). In this solution, the muscle often remained in good condition for as long as 12 hours, without development of the 'Ringer tonus' described by Winton (18).

The system used for rapid or gradual cooling of the smooth muscles is shown in figure 1 and operates as follows: Initially, water at 37°C. is pumped from bath *B* through the jacket, valves  $A_1$  and  $A_2$  being closed. Bath *A* is now set to any desired temperature by permitting water to enter it from one or the other of the coils passing through the tanks labelled 'Hot' and 'Cold.' Valves  $B_1$  and  $B_2$  are then closed, and valves  $A_1$  and  $A_2$  are opened, thus permitting water to pass from bath *A* into the jacket. At the same time, the interior of the muscle bath is flushed out with Tyrode's solution which has previously been cooled in a condenser to the temperature of bath *A*. The excess Tyrode's solution passes out an overflow adjusted so as to maintain a constant fluid level in the muscle bath. Cooling of the muscle from 37° to 5°C. can be accomplished in 15 to 25 seconds in this manner and to 0°C. in a somewhat longer time. When gradual cooling is desired, the water jacket is connected to bath *A* from the start, this bath then being cooled from 37°C. by permitting water to enter it from the coil in the tank kept cold by ice or refrigerant. For the nictitating membranes, the glass bulbs were connected in parallel and were located in the system in place of the jacket of the muscle bath. In this case, no flushing of the nictitating membranes with Tyrode's solution was employed.

The temperature of the smooth muscle in the bath was measured by means of a Western Electric rod-type thermistor, type 14B, located in the bath close to the lower end of the muscle, and connected through a Wheatstone bridge to a microammeter calibrated in degrees centigrade as described by Fastie and Drummeter (19). The temperature was recorded by following the pointer of the microammeter with a second pointer mechanically connected to a writing point moving vertically on the kymograph drum. Used in conjunction with a frontal writing lever for the muscle, this arrangement provided simultaneous records of muscle contractions and bath temperatures. A magnification factor ranging from 2 to 3 was used for the

isotonic muscle lever, which was loaded so as to provide a tension of approximately one to 2 gm.

### RESULTS

*Cooling as a Stimulus to Smooth Muscles.* The nictitating membranes of the cat and all other smooth muscles tested, including the retractor penis muscle and ureter of the dog, the intestine and uterus of the rat, and the intestine of the frog, contracted when cooled. As stated by Starling (12) with regard to the retractor penis muscle,

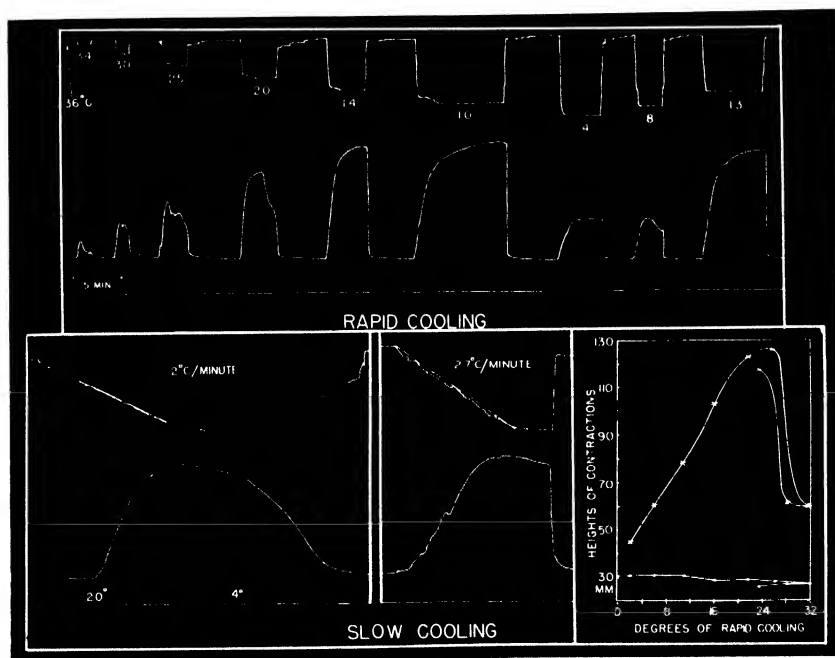


Fig. 2. CONTRACTIONS EVOKED BY RAPID OR GRADUAL COOLING, no other stimulus being used, retractor penis muscle of dog. Myograms below, temperatures above, in each record. *Lower right:* 'Cooling-response' curve, with degrees of rapid cooling plotted against heights of contraction. Resting lengths plotted below.

the contraction was greater with rapid cooling than with slow cooling through the same number of degrees. With rapid cooling, each of the various types of smooth muscle appeared to contract as soon after stimulation and at approximately as rapid a rate as when stimulated by drugs such as epinephrine or acetylcholine.

If maintained at the same temperature to which it was rapidly cooled, most muscles relaxed partially or fully within a period of a minute or two. This 'adaptation' or 'accommodation' to the cooling stimulus was more pronounced with moderate than with marked amounts of rapid cooling.

That it was the cooling that evoked the contraction, and not the mechanical stimulus resulting from flushing out the bath, was shown by the fact that no contraction followed flushing with Tyrode's solution at the initial temperature of 36° to 38°C.

Upon rewarming the muscle, prompt relaxation occurred, provided the peak of contraction had been reached or passed. If, however, the muscle was rapidly rewarmed from a temperature of  $5^{\circ}$  to  $15^{\circ}\text{C}$ . during the rising phase of the contraction, an additional spike-like contraction often occurred.

*'Cooling-Response' Curve.* When degrees of rapid cooling (abscissae) were plotted against heights of contraction, an S-shaped curve resulted. For the nictitating membranes of the cat, and the retractor penis muscle of the dog, it was found that this 'cooling-response' curve passed through a maximum at  $22^{\circ}$  to  $27^{\circ}$  of rapid cooling (from  $37^{\circ}$  to  $10^{\circ}$ – $15^{\circ}\text{C}$ .) and fell off sharply at more than  $30^{\circ}$  of rapid cooling (from  $37^{\circ}$  to  $7^{\circ}\text{C}$ . or less).

Figure 2 shows the contractions produced by increasing amounts of rapid cooling, the corresponding cooling-response curve, and 2 contractions resulting from gradual cooling. It will be noted, first, that a contraction was evoked by as little as  $2^{\circ}$  of rapid cooling, from  $36^{\circ}$  to  $34^{\circ}\text{C}$ ., and, second, that there was a marked adaptation or accommodation to this minimal stimulus, as indicated by the prompt relaxation of the muscle while still at  $34^{\circ}\text{C}$ .

That the falling off of the contraction with cooling to  $4^{\circ}\text{C}$ . was not due to deterioration of the preparation is shown by the subsequent cooling to  $13^{\circ}\text{C}$ ., with the resulting contraction only slightly less than those obtained previously with comparable stimuli.

With slow cooling, the rate of cooling determined not only the height of maximal contraction (which was frequently less than with rapid cooling, though this was not so in the myograms in figure 2 in which the contractions were nearly as high as with rapid cooling), but also the temperature at which the contractions started. Thus, with cooling at  $2^{\circ}\text{C}/\text{minute}$  no contraction occurred until a temperature of  $20^{\circ}\text{C}$ . was reached, but with cooling at  $2.7^{\circ}\text{C}/\text{minute}$  the contraction appeared almost immediately after cooling was started, at a temperature of  $33^{\circ}$  to  $34^{\circ}\text{C}$ . (fig. 2). With muscles which had deteriorated considerably as a result of long experimentation, the rate of cooling became a more significant factor, such muscles sometimes responding not at all to slow cooling, but still contracting with rapid cooling.

A striking feature of certain muscles, possibly as a result of deterioration, is the possible existence of a 'critical temperature' for contraction, a phenomenon which interested us particularly because of the critical temperature for vasoconstriction observed in the paws of cats or dogs (14).

In these muscles, rapid cooling from  $36^{\circ}$  to  $18^{\circ}\text{C}$ . might result in almost no contraction, but if the muscle was rewarmed and again cooled rapidly, this time from  $36^{\circ}$  to, say,  $16^{\circ}\text{C}$ ., it would show an almost maximal contraction. Why the second stimulus was so much more effective than the first is not clear. Perhaps, with certain rates of cooling, there was a critical temperature above which rapid cooling had little effect, and below which the muscle was powerfully stimulated.

*Sensitization of the Chronically Denervated Nictitating Membrane to Cooling.* By comparing, in the same animal, the response to rapid cooling of the chronically denervated nictitating membrane (n.m.) with that of the opposite, normal n.m., it was possible to determine whether denervation might actually sensitize the muscle to cooling, as it does to epinephrine. If it existed, such an effect might offer a possible

explanation for the sudden vasoconstriction observed in denervated or sympathectomized extremities exposed to cold (14). In 2 out of the 4 experiments such a sensitization did occur, the denervated n.m. showing a greater response for a given amount of cooling than the normal, for all less than maximal contractions, in which the 2 responses were essentially equal. (In the remaining 2 experiments there was no essential difference between denervated and normal.) Furthermore, the contrac-

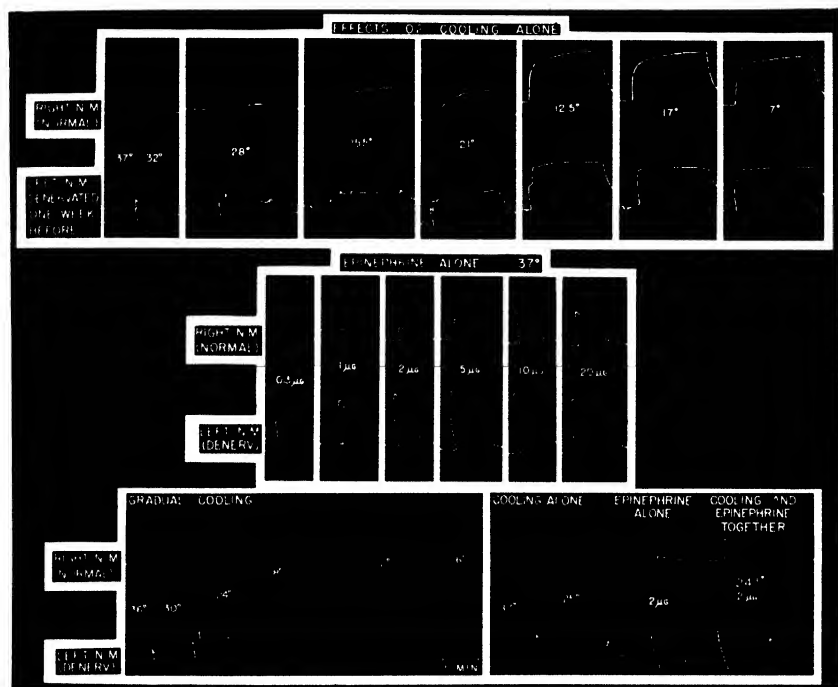


Fig. 3. CONTRACTIONS OF NORMAL (*upper tracings*) and CHRONICALLY DENERVATED NICITATING MEMBRANES OF CAT. Curarization maintained, adrenals removed and cervical sympathetic chain on normal side cut at start of experiment. *Upper record*: Rapid cooling from 37°C., the sole stimulus. *Middle record*: Epinephrine alone as a stimulus, nictitating membranes kept at 37°C. *Bottom record*: *left*, slow cooling; *right*, additive effects of the 2 stimuli, cooling and epinephrine, when applied simultaneously.

tions of the denervated n.m. were step-wise and jerky, in contrast to the smooth contraction of the normal, and the latency between the time of application of the cooling stimulus and the start of the response was often greater for the denervated than for the normal n.m. (fig. 3). The denervated n.m. was, incidentally, found to be more responsive to the stimulus of sudden stretching than the normal.

*Cooling Apparently Acts on a Different System than Epinephrine.* The response to the cooling stimulus differs from the response to epinephrine in several respects: *a*) In the denervated nictitating membrane, the shape of the curve of contraction with cooling was step-like, in contrast to the smooth contraction produced by epinephrine. *b*) The response of the denervated nictitating membrane to increased

amounts of cooling differed from its response to increasing amounts of epinephrine. Unfortunately, one cannot compare the shapes of the curves of response to the 2 stimuli, since degrees of rapid cooling cannot be considered equal, say, to  $\mu\text{g}$ . of epinephrine. However, the response curves do emphasize certain contrasts between the 2 stimuli, as follows: 1) the response of the denervated n.m. to cooling was erratic, with scattered points, whereas the response of the same muscle to epinephrine followed a very smooth curve (fig. 4); 2) these scattered points for the denervated n.m. fall above and to the left of the curve for the normal with small amounts of cooling, but with large amounts they lie below and to the right; 3) with epinephrine the dose-response curve for the denervated n.m., whose points were obtained alternately with those for the cooling-response curve, falls consistently above and to the

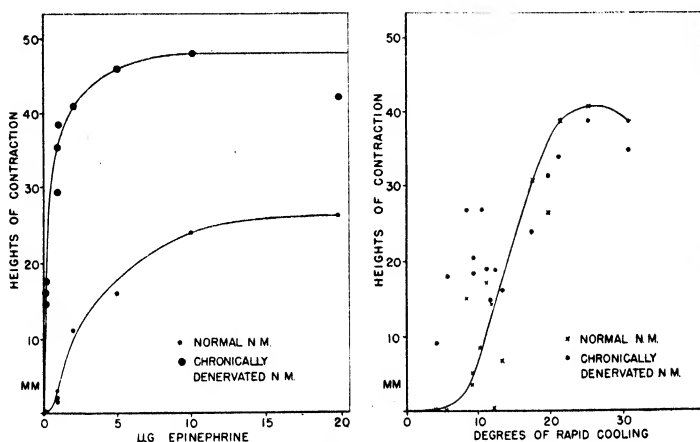


Fig. 4. COOLING-RESPONSE CURVES (*right*) for normal and chronically denervated nictitating membranes for experiment shown in figure 3, showing sensitization of denervated nictitating membrane to the cooling stimulus. No attempt was made to connect the points of the denervated nictitating membrane. EPINEPHRINE DOSE-RESPONSE CURVES (*left*), same experiment.

left of the curve for the normal n.m. (fig. 4). *c*) The adrenolytic drug, Dibenamine, abolishes the excitatory action of epinephrine, the latter then tending to produce lengthening of the retractor penis muscle, but cooling still causes shortening. *d*) The effects of cooling and of epinephrine, when applied simultaneously, are roughly equal to the sum of their separate effects. Numerous experiments have been done in which the muscle was first stimulated by cooling alone, then by epinephrine alone, and finally by both together, using the same amount of each as previously. Before Dibenamine, the simultaneous effects of cooling and epinephrine were roughly additive, producing a greater contraction than either singly, as shown for the nictitating membrane in figure 3 (*right, lower*) and for the retractor penis in figure 5 (*upper*).

After Dibenamine the combined effects of cooling and epinephrine were less than cooling alone, indicating algebraic addition, with the epinephrine tending to produce lengthening, i.e. a 'minus contraction' (fig. 5, *upper, right*). Actual lengthening with epinephrine alone has been observed in retractor penis muscles possessing

a certain amount of resting 'tone,' so that they are not prevented by their connective tissue components from lengthening. When the intensity of the 2 stimuli, cooling and epinephrine, are suitably graded after Dibenamine, their combined effect may be zero, as a result of the cancelling out of these 2 opposite effects.

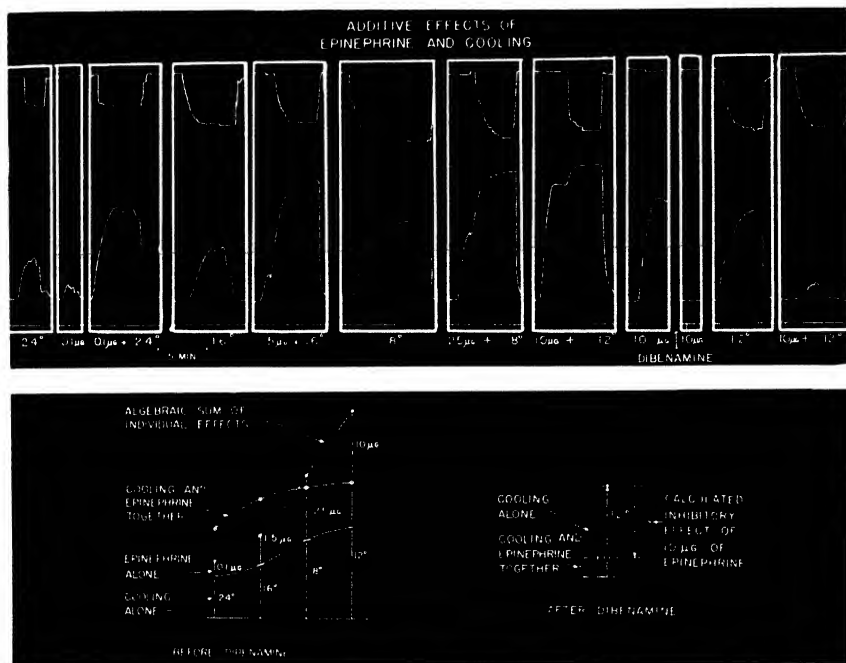


Fig. 5. ADDITIVE EFFECTS OF COOLING AND EPINEPHRINE. *Upper:* Cooling and epinephrine were first tested separately and then the same amounts of each were applied simultaneously. On the later tracings the muscle was cooled just after the peak of the contraction produced by epinephrine alone. Following Dibenamine, epinephrine produced no visible effect, but cooling still evoked a contraction. With both stimuli together, the contraction was less than with cooling alone, epinephrine now tending to produce lengthening or a 'minus contraction.' *Lower left:* Diagrammatic representation of above data. Theoretical sum of the 2 separate effects shown by dotted line; actual response to both, solid line. Apparent synergism at lower intensities of stimulation probably due to preservation of epinephrine at the lower temperatures used when the 2 stimuli were applied together. Distances on abscissae are arbitrary. *Lower right:* After Dibenamine the line indicating the effect of epinephrine is directed downward, indicating a 'minus contraction,' which is thus added algebraically to the contraction produced by cooling.

Curves have been constructed to show the algebraically additive effects of the 2 stimuli, both before and after Dibenamine (fig. 5, *lower*). The apparent synergism existing with small doses of epinephrine is almost certainly due to the fact that epinephrine in Tyrode's solution is better preserved, and thus has a greater effect, at the lower temperatures used when both stimuli were applied simultaneously, than at the temperatures of 36° to 38°C. used for studying the effects of epinephrine alone.

*Responses of Spontaneously Rhythmic Muscles to Cooling.* 1) *Uterus, pregnant rat.* Contractions of large amplitude characterized the spontaneous rhythm of the

several specimens of uteri from pregnant rats which were studied. The effect of cooling through a few degrees was to decrease the amount of relaxation, with little

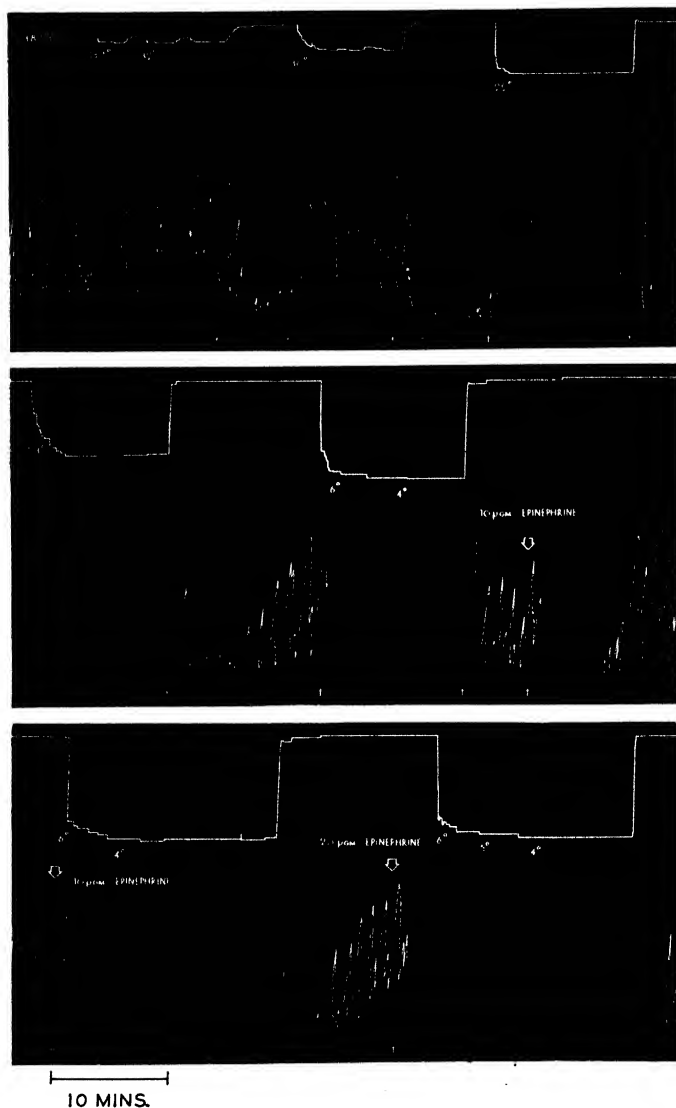


Fig. 6. RAPID COOLING OF UTERUS of pregnant rat. The inhibitory or 'minus contraction' effect of epinephrine is shown in the lower record, the contractions due to cooling to  $4^{\circ}\text{C}.$ , plus epinephrine, being less than the contraction due to cooling to  $4^{\circ}\text{C}.$  alone (middle record).

increase in height of contraction, and to slow the rhythm (fig. 6, top). With more marked cooling there was a smooth contraction completely devoid of all signs of

rhythmicity (fig. 6, *middle*). Algebraic addition of the effects of cooling and of epinephrine was readily demonstrated on the uterus. Since epinephrine characteristically produces an inhibitory effect on this organ, it was not surprising that with cooling and epinephrine together the height of the contraction was less than with cooling alone (fig. 6, *bottom*).

2) *Intestine, rat*. In the several preparations studied, the spontaneously rhythmic contractions were small. With rapid cooling through a few degrees, the response consisted of a rise in the baseline with little change in the height of the superimposed rhythmic contractions. With larger amounts of rapid cooling, there was prompt contraction, many times higher than the original rhythmic contractions, which usually disappeared completely under these circumstances.

An abrupt contraction, usually accompanied by an increase in rhythmicity, was found to follow rewarming of the majority of the preparations of intestine studied. Such a contraction is shown in figure 7 following cooling to 6°C. That this contraction was not caused by a stimulating effect produced by rapid rewarming per se is shown by the failure of frequent rapid rewarming to produce a contraction (fig. 7, *lower, extreme right*).

3) *Ureter, dog*. One or 2 contractions, about as high as the spontaneously rhythmic contractions, were elicited by rapidly cooling the ureter, which then became unresponsive and lost its spontaneous rhythm.

4) *Intestine, frog*. With rapid cooling from 37°C. all the way down to 10°C. in some preparations, not only were the large rhythmic contractions of the frog intestine partially inhibited, but also there was an actual lengthening of the muscle. With more marked cooling, the muscle shortened, the spontaneous rhythm slowing markedly in rate or disappearing entirely.

5) *Enhancement of rhythmicity in retractor penis muscle by cooling*. In most experiments the retractor penis muscle showed no spontaneous rhythm at 36° to 38°C., but with rapid cooling small rhythmic contractions often appeared in the rising portion of the curve of contraction, and large rhythmic contractions often appeared when the muscle was kept at 25°C., which seemed, in several preparations, to be the optimal temperature for the appearance of rhythmicity. Spontaneous rhythmicity was most marked in muscles which were not removed from the animal until 15 to 30 minutes after death.

#### DISCUSSION

The question arises as to how and where cooling acts as a stimulus to smooth muscles. The studies in which Dibenamine reverses the action of epinephrine, but not that of cooling, suggest certain possibilities: 1) that cooling acts on an entirely different excitatory system than epinephrine; or 2) that it acts on the same excitatory system, but at a lower link than epinephrine in a chain of events leading to excitation; or 3) that it acts directly on the contractile system. These studies with Dibenamine make it unlikely that cooling produces excitation by stimulating the nerves in the retractor penis muscle, because stimulation of the adrenergic nerves to the muscle would almost certainly cause lengthening after Dibenamine just as epinephrine does, and stimulation of cholinergic nerves, presumably unaffected by Dibenamine, would also cause lengthening.



The difference between the action of the 2 stimuli is further emphasized by the differences in the contraction and response curves of the denervated nictitating membrane with cooling as a stimulus on one hand, and epinephrine on the other. Another contrast is provided by the prompt adaptation or accommodation of the

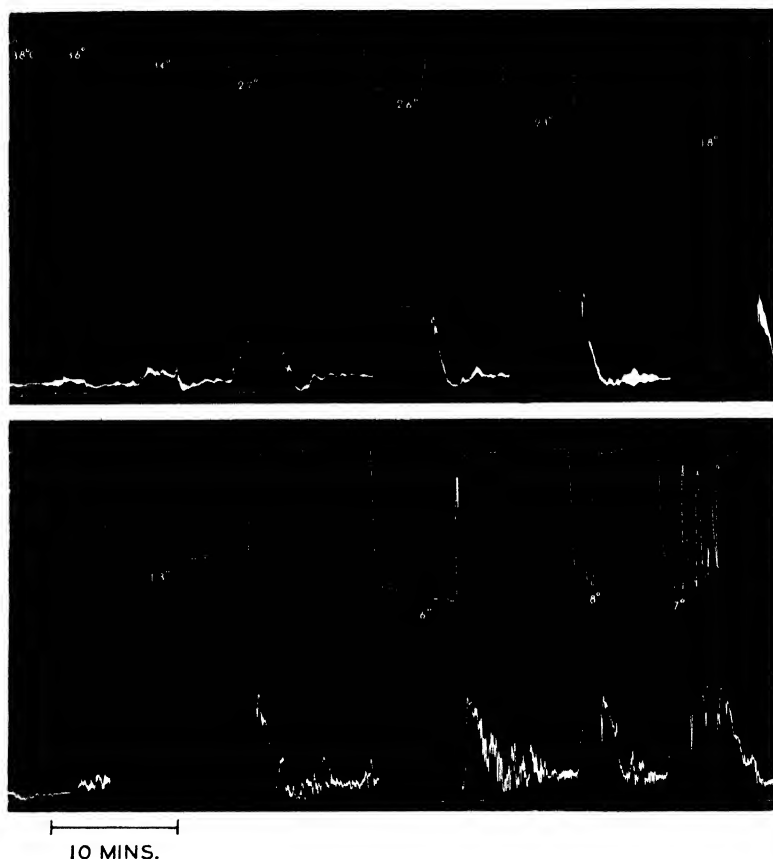


Fig. 7. RAPID COOLING OF INTESTINE of rat. Upon rewarming after prolonged cooling a spike-shaped contraction, with superimposed rhythmic waves, ensues, but no contractions follow rapid rewarming after brief periods of cooling (*bottom, extreme right*).

muscle to the cooling stimulus, but not to epinephrine, to constant concentrations of which there is usually a steady, undiminishing response.

In the hope of learning whether cooling acts via an excitatory system (propagation presumably present) or directly on the contractile system (no propagation, response termed 'contracture' according to Gasser, 20), we are engaged in cooling one end of the muscle in order to determine whether or not the contraction spreads to the other end. We shall also attempt later to record action potentials from smooth muscles stimulated by cooling.

A distinction has long been made between the phasic, quick type of contraction of smooth muscle and the tonic, slow type. In spontaneously rhythmic muscles, the primary contractions are the phasic type and are superimposed on a slowly shifting base line representing the tonic type of contraction. Winton (18, 21) has shown that phasic contractions, which can be produced by epinephrine, or by 60-cycle a.c. stimulation of the retractor muscle of *Mytilus edulis*, were accompanied by a decreased viscous resistance, or 'viscosity' of the muscle, whereas tonic contractions, which were produced by d.c. stimulation were accompanied by an increased viscous resistance. Inasmuch as Winton (18) also found an increase in viscous resistance with cooling, it may be that the contraction evoked by cooling is of the tonic type. In the hope of clarifying this point, and of learning more about the other characteristics of the contraction evoked by cooling, we are now studying the mechanical properties of smooth muscles contracting on one hand to cooling, and on the other hand to epinephrine or other stimuli.

One is tempted to ask whether or not the cooling stimulus acts by virtue of a gradient of temperature set up between the inner and outer portions of the muscle. Certain of the observed facts tend to support this idea, including: 1) the spike-like contraction observed following rapid rewarming of a cooled muscle during the rising phase of contraction. This contraction could occur as a result of a sudden reversal of the temperature gradient, the inside of the muscle now being cooler than the outside. The direction of the temperature gradient, i.e. either inward or outward, should probably make little difference, owing to the microscopic dimensions of the individual smooth muscle cells; 2) the observed adaptation, or accommodation to rapid cooling could be caused by the disappearance of a temperature gradient within the muscle when the temperature is held constant after cooling. Similarly, 3) the usually lowered effectiveness as a stimulus of gradual cooling as compared with rapid cooling could be the result of a corresponding decrease in the temperature gradient.

On the other hand, there is evidence that though a gradient of temperature within the muscle may well be one of the factors leading to contraction, the absolute temperature of the muscle may also be a factor. Thus, first, in some instances, even with very gradual cooling, a nearly maximal contraction did finally occur, and, second, a contraction has only occasionally been observed to occur when the muscle was rapidly warmed during the phase of partial or complete relaxation occurring, as a result of adaptation, when the temperature was held constant after cooling.

Finally, inasmuch as smooth muscles may be stimulated by solutions containing excess potassium or sodium (22), cooling might be acting as a stimulus by changing the velocity of certain chemical reactions or equilibria so as to affect the concentrations of one or both of these, or of other ions. A subsequent back diffusion of ions from a region close to the cell membrane toward one farther away could explain the observed adaptation or accommodation to the cooling stimulus.

#### SUMMARY

Rapid or slow cooling acts as a stimulus to contraction for all the smooth muscles so far tested, including the nictitating membrane of the cat, the ureter and retractor penis muscle of the dog, the intestine and uterus of the pregnant rat, and with

cooling below approximately  $10^{\circ}\text{C.}$ , the intestine of the frog. When degrees of rapid cooling are plotted against heights of contraction, one obtains an S-shaped 'cooling-response' curve, which falls off with extreme degrees of cooling. The muscle 'adapts' or 'accommodates' to the cooling stimulus, as indicated by partial relaxation at constant temperature after rapid cooling and by a lessened response observed with gradual cooling. In 2 out of 4 experiments, chronic denervation of the nictitating membrane made it more sensitive to cooling as well as to epinephrine, the denervated nictitating membrane contracting more with a given amount of cooling than the normal for all less than maximal contractions.

Several lines of evidence indicate that, although the mode of action of cooling as a stimulus is unknown, cooling does act on a different excitatory system than epinephrine, or else acts directly on the contractile mechanism, as follows: *a*) In the retractor penis muscle, the adrenolytic agent, Dibenamine, reverses the action of epinephrine, which then tends to produce lengthening, but cooling still elicits a contraction. *b*) The separate effects of epinephrine and of cooling are roughly additive when applied simultaneously, but after Dibenamine the effects tend to cancel (algebraic addition). *c*) The contraction and response curves obtained for the denervated nictitating membrane with cooling differ from those obtained with epinephrine.

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## RELEASE OF GASTRIN IN RESPONSE TO BATHING THE PYLORIC MUCOSA WITH ACETYLCHOLINE<sup>1, 2</sup>

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**S**UGGESTIVE evidence indicates that the release of gastrin may be, at least in part, under nervous control. This evidence consists of the demonstration that certain drugs such as atropine, cocaine and procaine, which characteristically exert their effect on neural mechanisms, influence the release of gastrin. Atropine in moderate doses completely abolishes the secretion of acid in response to the gastric phase of gastric secretion in dogs (1). Two kinds of stimuli are effective in causing gastrin release, namely, distention and secretagogues. The action of both secretagogues and distention is counteracted by atropine (2). Since atropine does not significantly alter the response to injections of gastrin extracts (3), it has been reasoned that atropine acts by preventing the release of gastrin rather than by counteracting its action once it has been released (4).

Topical application of solutions of cocaine or procaine to the mucosa of a gastric pouch renders it unresponsive to stimulation by distention or secretagogues (5). That these local anesthetics do not act by paralyzing the parietal cells is shown by the fact that the response to histamine is unaltered and even more clearly by studies in animals with 2 gastric pouches, pyloric and fundic. In such animals application of a secretagogue to the pyloric mucosa causes acid secretion by the fundic glands. Local anesthetization of the pyloric mucosa prevents this effect but local anesthetization of the fundic mucosa does not (5). Therefore, with local anesthetics, as with atropine, the most plausible explanation for their blocking action is that they interfere with the release of gastrin.

With these indications for the participation of a neural mechanism at hand, further information on the question might be gained with the use of autonomic pharmacologic agents by attempting to stimulate rather than block gastrin release. We were quickly able to demonstrate that irrigation of the pyloric mucosa with acetylcholine solution caused the fundic glands to secrete acid. Further experiments were then made which demonstrate that this effect was probably due to release of gastrin from the pyloric mucosa.

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## METHODS

In 3 dogs the stomach was isolated by transection at the esophago-gastric junction and at the pyloric sphincter. The vagus nerves were cut. Alimentary tract continuity was restored by esophago-duodenal anastomosis. The stomach was then transected into a pyloric and fundic portion, the former deriving its blood supply from the lower branches of the left gastric artery, the latter from the upper branches of the left gastric and from the left gastro-epiploic vessels. The transection incisions and the cardia were sutured closed and a metal cannula was introduced into the fundic portion, wrapped in omentum, and brought out through a stab wound on the left side of the abdomen. The pyloric end of the pyloric pouch was brought through another stab wound on the right side to serve as a stoma. The 'pyloric' pouch included all of the pyloric mucosa plus a significant portion of the fundic mucosa.

Juice was collected only from the fundic pouch. Volume was recorded and acidity was determined by titration with 0.03 N NaOH using *p*-dimethylaminoazobenzene and phenolphthalein as indicators. Pepsin was determined by our modification (6) of Anson's hemoglobin method (7).

## RESULTS

*Introduction of Acetylcholine Solution into Pyloric Pouch.* In order to irrigate the pyloric pouch a self-retaining catheter was introduced into the stoma of the pouch. The end of the catheter was connected by a piece of rubber tubing to a reservoir funnel attached by a clamp to the side of the stock in which the dog was held. The pouch was irrigated for 30 minutes with 50 cc. of 0.5 per cent acetylcholine bromide<sup>3</sup> in physiological sodium chloride solution at 5 to 10 cm. of perfusion pressure. If any of the fluid leaked from the pouch during this time, enough was added to the funnel to maintain the fluid level at the original height. Samples of gastric juice were collected from the fundic pouch for 2 10-minute periods before perfusion (basal control), 3 10-minute periods during perfusion and 3 10-minute periods following perfusion. The average results for acid secretion of 15 such tests, 5 on each of the 3 dogs, are given in table 1, and the results of one such experiment are presented in figure 1. On the average, stimulation of acid secretion began during the second 10-minute period of irrigation, continued to increase during the next 3 periods, reached a peak during the second 10-minute period after cessation of irrigation, and then gradually returned to the basal level. The average magnitude of the response was more than 1½ times as great as the response to a moderate dose of histamine given subcutaneously (0.07 mg. of the diphosphate/kg.; table 2).

*Introduction of Saline Solution into Pyloric Pouch.* In control experiments, physiological saline without acetylcholine was used as the irrigating fluid; stimulation of acid secretion did not occur (table 1). This indicates that the irrigation procedure did not produce enough distention to cause gastrin to be released, which is known to occur when adequate distention is employed (8).

*Administration of Acetylcholine by Other Routes.* Having demonstrated that acetylcholine (ACh) application to the pyloric mucosa caused the fundic glands to secrete acid, it was next determined whether the effect was due to the absorption of ACh into the blood stream followed by direct action of ACh on the parietal cells, or to the release of gastrin from the pyloric mucosa by the local action of ACh. The fact that symptoms such as retching and salivation did not occur during irrigation with

<sup>3</sup> Kindly supplied by Dr. R. Pogge of Merck & Co., Inc.

the ACh solution indicated that significant amounts of ACh were not being absorbed. More direct evidence on this point was gained by demonstrating that administration of an amount of ACh solution equal to that used in the pyloric irrigation experiments failed to stimulate secretion by the fundic pouch when introduced into the rectum, the duodenum, or directly into the fundic pouch or when injected subcutaneously. The results of these experiments are given in table 1. In no instance did significant stimulation of gastric secretion occur. The results of subcutaneous injection are in agreement with those of other workers (9, 10) who found that doses of the order of 400 mg. of ACh subcutaneously produced only small secretory responses.

TABLE 1. ACID OUTPUT FROM FUNDIC POUCH IN RESPONSE TO ACETYLCHOLINE, SALINE, AND NICOTINE

TABLE I. ACID OUTPUT												
DRUG	CONC. AND DOSAGE	ROUTE OF ADMINISTRATION	NO. OF TESTS	HCl OUTPUT (mm/10-MINUTE PERIOD)								
				Before drug		During irrigation			After drug			
				1	2	1	2	3	1	2	3	
ACh	% 0.5	Irrigation of pyloric pouch	15	0.004 ±0.0028	0.009 ±0.0047	0.010 ±0.0046	0.124 ±0.0175	0.247 ±0.0179	0.503 ±0.0126	0.822 ±0.0154	0.061 ±0.0115	
NaCl	0.9	Irrigation of pyloric pouch	15	0.014 ±0.0009	0.002 ±0.0014	0.013 ±0.0129	0.013 ±0.0040	0.028 ±0.0119	0.029 ±0.0144			
ACh	50 cc. 0.5	Oral <sup>1</sup>	3	0.0	0.006				0.012	0.006	0.003	
ACh	50 cc. 0.5	Rectal	3	0.015	0.024				0.003	0.0	0.067	
ACh	50 cc. 0.5	Subcutaneous	3	0.021	0.018				0.021	0.018	0.015	
ACh	0.5	Irrigation of fundic pouch	3	0.012	0.012				0	0	0	
Nicotine	0.5	Irrigation of pyloric pouch	5	0.006	0.003	0.006	0.006	0.006	0.012	0.012	0.012	

Values following means are standard errors.

<sup>1</sup> Since these animals had an esophago-duodenostomy, oral administration was essentially equivalent to intra-duodenal administration.

These findings all point toward the pyloric mucosa as a special site for the action of ACh in stimulating acid secretion and against absorption into the blood stream as the mechanism of its action.

*Pepsin Content of Juice Secreted in Response to Acetylcholine, Histamine and Urecholine.* As further evidence that the juice secreted was not the result of ACh in the blood the pepsin content of the juice secreted in response to irrigation of the pyloric mucosa with ACh solution was compared with that evoked by subcutaneous injections of histamine or the parasympathomimetic drug Urecholine<sup>4</sup> (urethane of beta methylcholine). The results are presented in table 2 and show that ACh solution in the pyloric pouch produced a juice very low in pepsin, even lower than with histamine

<sup>4</sup> Kindly supplied by Dr. R. Pogge of Merck & Co., Inc.

stimulation and far lower than that resulting from Urechole injection. This evidence supports the view that ACh stimulated acid secretion by releasing gastrin rather than by acting directly on the fundic glands after absorption. Furthermore, the findings are in keeping with the previous demonstration from this laboratory (11) that release of gastrin by irrigation of the pyloric mucosa with a chemical secretagogue (liver extract) causes the fundic glands to secrete a pepsin-poor juice. Our findings also conform with the fact that injection of gastrin extracts produces a juice of low-pepsin content (3).

*Introduction of Nicotine into Pyloric Pouch.* To determine whether the action of ACh on the pyloric mucosa was muscarinic or nicotinic, irrigation experiments were performed in which a 0.5 per cent solution of nicotine sulfate was substituted for ACh. No stimulation occurred (table 1), which indicates that this action of ACh is

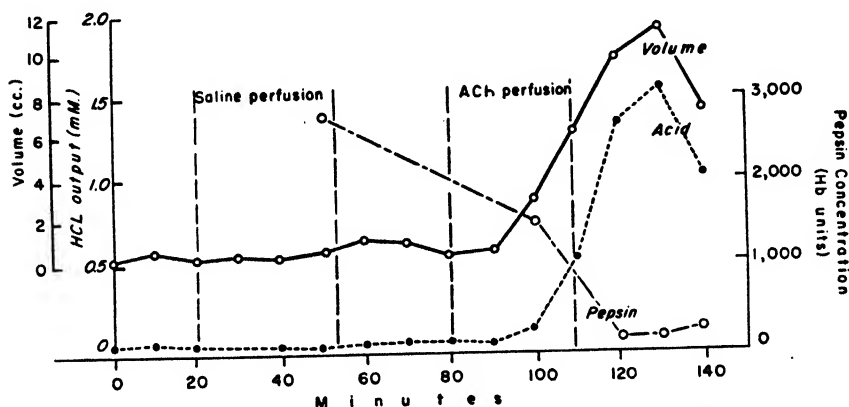


Fig. 1. VOLUME, ACID AND PEPSIN RESPONSE of the fundic pouch to irrigation of the pyloric pouch with saline and with 0.5 per cent acetylcholine solution in a dog with two pouches.

probably muscarinic, as would be anticipated by the fact that it is counteracted by atropine.

#### DISCUSSION

The results of this study can be considered as strong evidence in support of the idea that a local nervous mechanism with cholinergic endings is concerned in the release of gastrin by the pyloric mucosa. One would postulate a nervous arrangement in which the nervous receptors on the mucosal surface are responsive to chemical (secretagogue) or mechanical (distention) stimulation. These receptors can be inactivated by anesthetization with cocaine. Activation of these receptors leads to a cholinergic discharge at the neuroeffector junction with the cell forming gastrin (probably the mucoid cell of the pyloric glands as first suggested by Lim (12)). This cholinergic discharge releases gastrin, an effect which was mimicked in the present experiments by direct application of the ACh to the pyloric mucosa. It should be noted that according to this scheme the site of action of ACh is not the same as that of secretagogues. Atropine would, of course, block the action of acetylcholine on the

gastrin-forming cell. It must be pointed out that this scheme is derived wholly from pharmacologic and physiologic observations, there being no clear anatomic evidence for the existence of nerves such as those described. There are, of course, nerve fiber and cell bodies in the substantia propria and submucosa of the gastric glands but these have not been demonstrated to have connections such as we postulate.

Are there any extrinsic nerves which activate this hypothetical intrinsic neural mechanism? The interpretation of the findings of the present study is considerably altered depending on whether the question "Do vagal nervous impulses release gastrin?" is answered affirmatively or negatively. Uvnäs (13) has claimed that the gastric acid response to electrical stimulation of the vagi is greatly reduced by pyloric extirpation. This was interpreted as indicating that the vagi release gastrin from the pyloric mucosa. However Babkin and coworkers (14, 15) have repeated Uvnäs' experiments and arrived at different conclusions. They found that the acid secre-

TABLE 2. VOLUME, ACID OUTPUT, PEPSIN CONCENTRATION AND PEPSIN OUTPUT OF FUNDIC POUCH IN RESPONSE TO IRRIGATION OF PYLORIC POUCH WITH 0.5 PER CENT ACETYLCHOLINE BROMIDE SOLUTION IN PHYSIOLOGICAL SALINE, AND IN RESPONSE TO SUBCUTANEOUS INJECTIONS OF HISTAMINE OR URECHOLINE

DRUG	ROUTE OF ADMINISTRATION	CONC. AND DOSAGE	NO. OF TESTS	VOLUME	HCl OUTPUT	PEPSIN CONCENTRATION	TOTAL OUTPUT OF PEPSIN
				cc/30 min	mM/30 min	Hb. U/cc.	Hb. U/30 min
ACh	Irrigation of pyloric pouch	0.5% in saline	15	19.6±2.35	2.188±0.36	280±68	4,104±4,000
Histamine	Subcutaneous	0.07 mg/kg.	13	12.0±3.6	1.320±0.236	732±126	10,445±2,500
Urecholine	Subcutaneous	0.07 mg/kg.	14	11.7±1.50	1.257±0.209	2,136±255	35,483±4,500
NaCl	Irrigation of pyloric pouch	0.9%	10	2.5±0.58	0.105±0.039	2,571±692	6,125±1,600

tion in response to electrical stimulation of the vagi was only transiently lowered by pyloric extirpation and they attributed the drop to the effect of surgical trauma. Furthermore, as has been pointed out elsewhere (4), experiments have been performed which indicate that vagal stimulation does not release gastrin. These consist of the demonstration that sham feeding and insulin hypoglycemia, which act as powerful stimulants to gastric secretion and are dependent solely upon vagal activity, fail to cause secretion in the Heidenhain (vagally denervated) pouch at a time when the main stomach is strongly activated (16). If gastrin release occurred with the secretory response in the main stomach, the Heidenhain pouch would be expected to respond to this circulating hormone just as it does when gastrin is released by the presence of food in the main stomach. However, the possibility must be considered that vagal nervous stimulation releases an amount of gastrin which is too small to stimulate the Heidenhain pouch but is large enough to stimulate the fundic glands 'sensitized' by the cholinergic impulses from the vagi. There is good evidence to show that the vagally innervated fundus is more responsive to gastrin



than the vagally denervated fundus (4). If we assume that vagal stimulation can release gastrin we must also assume that the amount of gastrin which it releases is less than that produced by pyloric distention or irrigation of the pyloric mucosa with secretagogues or with acetylcholine because the latter are capable of strongly stimulating the Heidenhain pouch. The theory that vagal impulses release gastrin cannot be considered to be established until some experiment has been devised to detect the circulating gastrin which is presumed to be present. Meanwhile no final conclusion can be reached as to whether or not the local cholinergic mechanism for gastrin release has extrinsic connections.

It is clear from the work of both Uvnäs and Babkin that vagal impulses can still stimulate some acid secretion after the pyloric portion of the stomach has been removed, although Uvnäs stated that this procedure decreased the response and Babkin found that it did so only transiently. What is the mechanism of this vagal stimulation of acid secretion after pyloric extirpation? The vagus nerves to the stomach are known to be cholinergic (17), but pharmacologic parasympathomimetic agents, when confined to the fundic glands, fail to stimulate acid secretion. The latter statement is based upon the studies of Morton and Stavrakys (18) and of Uvnäs (19) who found that intra-arterial injection of ACh failed to stimulate acid secretion. Our results with irrigation of the fundus with ACh solution also indicate failure of ACh to act directly on the parietal cell. Although ACh probably does not act as a direct stimulant to the parietal cell, there is evidence to indicate that it sensitizes the parietal cell to stimuli which are capable of acting directly (4).

How, then, do vagal nerve impulses stimulate acid secretion—which they certainly do? No completely satisfactory answer can be given at the present time but the following theoretical possibilities suggest themselves: *a*) the postganglionic vagal endings on the parietal cell release, in addition to ACh a second, as yet unidentified, neurohumoral agent, an X-substance, capable of acting directly on the parietal cell; *b*) vagal impulses are capable of releasing gastrin from some site other than the pyloric mucosa, but this possibility is faced with the same difficulties already stated for gastrin release from the pyloric mucosa by vagal impulses.

Babkin's (20) suggestion that the ACh released by the postganglionic vagal endings in turn causes release of histamine from the gastric mucosa would fail to explain the inability of ACh to stimulate when injected intra-arterially or when irrigated onto the surface of the fundic glands.

This study indicates that the parenterally administered stable choline esters and other parasympathomimetic drugs stimulate acid secretion by releasing gastrin from the pyloric mucosa. It has also been found (22) that surgical extirpation of the pyloric pouch causes a profound decrease in the acid secretory response to parasympathomimetic drugs of the fundic pouch of these 2-pouch dogs.

The results of the present study may be interpreted as indicating that the pyloric mucous membrane is the most important site for gastrin formation, thus raising the question of whether or not any other sites exist. We have considered this question extensively elsewhere (4).

Brooks and coworkers (21) have recently demonstrated that intragastric instillation of ACh solution is a strong stimulus for acid secretion in the human. They, however, postulated a direct action of ACh on the acid-forming cells.

## SUMMARY

In dogs with the stomach divided into 2 isolated pouches (pyloric and fundic), irrigation of the pyloric pouch with 0.5 per cent solution of acetylcholine bromide produced strong stimulation of acid secretion by the fundic glands. This juice was low in pepsin content like that secreted in response to histamine injection and unlike the high-pepsin juice which resulted from stimulation with Urecholine injected subcutaneously. When the acetylcholine solution was given by subcutaneous injection or by instillation into the duodenum, rectum or fundic pouch, acid secretion was not stimulated. Irrigation of the pyloric pouch with physiological saline solution or with 0.5 per cent solution of nicotine sulfate also failed to stimulate acid secretion.

## CONCLUSION

Topical application of acetylcholine solution to the pyloric mucosa causes gastrin to be released, resulting in stimulation of acid secretion by the fundic glands. The relationship of this finding to the mechanism of stimulation of acid secretion by vagal impulses is discussed.

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# EFFECT OF CINCHOPHEN UPON EXTERNAL SECRETION OF THE PANCREAS

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IT HAS been shown (1) that cinchophen administered orally decreases the volume and concentrations of both alkali and mucin in the juice secreted by the first part of the duodenum. Cinchophen likewise has been shown to diminish the volume of juice secreted from the dog's pyloric pouch (2). That cinchophen is a potent choleric has long been known (3).

Churchill and Van Wagoner (4) established that peptic ulceration occurring in either the stomach or duodenum is an almost invariable sequel to severe cinchophen intoxication in the dog. Pancreatic secretion being alkaline in reaction may therefore be a factor of importance in the production or prevention of this symptom.

## METHODS

Pancreatic juice was collected over 15-minute periods from the cannulated pancreatic duct of dogs anesthetized with pentobarbital. The output of juice was stimulated throughout the entire experiment by infusing a dilute solution of secretin<sup>2</sup> (0.15 mg/cc.) at a rate of 20 cc. per 15 minutes into the animal's blood stream by way of the femoral vein. It had been previously determined that for the preparation and the solution used this rate of infusion, while insuring a constant moderate secretion, was submaximal. Five of the dogs were fed cinchophen in daily amounts of 200 mg/kg. for 4 days before the commencement of the acute phase of the experiment; for brevity these are known as the 'chronic' animals. The other animals received only a normal diet prior to the acute phase and are hence designated as 'acute.'

After collecting three 15-minute samples, agreeing within 0.2 cc., 100 mg/kg. of sodium cinchophen was injected by way of the femoral vein in each trial and further 15-minute collections of juice were made. The sodium cinchophen was prepared by dissolving white powdered cinchophen in 5 per cent sodium bicarbonate. The resulting solution was brought to approximately pH 7.4 and then injected. The alkali content of all the samples of juice was determined by adding an excess of N/10 hydrochloric acid and titrating the excess over the alkali of the juice with sodium hydroxide. The alkalinity of the secretion was calculated in terms of cc. of N/10 sodium hydroxide.

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<sup>1</sup> Rockefeller fellow.

<sup>2</sup> The secretin was kindly supplied by Dr. L. L. Gershbein.

## RESULTS

Tables 1 and 2 show the results obtained in both the 'acute' and 'chronic' preparations, respectively. Only *dog 1* in the 'acute' series showed any decrease in the volume or alkali output. In all other cases, an increase in both was noted. The

TABLE 1. 'ACUTE' EXPERIMENTS

DOG	MEAN OF THREE 15-MINUTE COLLECTION PERIODS BEFORE CINCHOPHEN		MEAN OF THREE 15-MINUTE COLLECTION PERIODS FOLLOWING CINCHOPHEN		CHANGE	
	Volume	Alkali Content	Volume	Alkali Content	Volume	Alkali
	cc.	cc. N/10 NaOH	cc.	cc. N/10 NaOH	%	%
1	2.7	5.19	2.56	4.35	-5.1	-16
2	2.20	3.98	4.16	8.14	+88.1	+104.5
3	2.56	4.33	4.66	9.40	+83.9	+117.1
4	1.53	2.19	2.43	4.13	+58.8	+88.5
5	1.53	2.38	2.20	3.86	+43.7	+62.1
6	1.06	1.30	2.10	3.82	+98.1	+194.8
7	1.76	1.86	3.16	4.86	+79.8	+161.6
8 <sup>1</sup>	0.91	0.55	2.26	5.43	+148.3	
Mean	1.91	3.03	3.32	5.50	+64 <i>P</i> < 0.01	+87.5 <i>P</i> < 0.02

<sup>1</sup> This result discarded on account of abnormally low control volume.

TABLE 2. 'CHRONIC' EXPERIMENTS

DOG	MEAN OF THREE 15-MINUTE COLLECTION PERIODS BEFORE CINCHOPHEN		MEAN OF THREE 15-MINUTE COLLECTION PERIODS FOLLOWING CINCHOPHEN		CHANGE	
	Volume	Alkali content	Volume	Alkali content	Volume	Alkali
	cc.	cc. N/10 NaOH	cc.	cc. N/10 NaOH	%	%
1	3.17	4.41	2.50	3.75	-21.1	-14.9
2	5.66	10.28	5.90	11.18	+4.2	+8.6
3	1.30	1.36	1.53	1.52	+17.6	+11.7
4	2.86	3.38	4.26	6.01	+48.9	+77.8
5	4.53	3.73	6.00	5.60	+31.5	+44.8
Mean	3.50	4.63	4.04	5.60	+16.2 <i>P</i> > 0.05	+25.6 <i>P</i> > 0.05

average increase in volume was 64 per cent and in the alkali content of the juice 87.5 per cent. Both these figures are statistically significant.

In the 'chronic' series only *dog 1* showed a decrease in volume and alkali following the injection of cinchophen. The overall average increase was 16.2 per cent in volume and 25.6 per cent in alkali. Neither of these figures is statistically significant. Cinchophen did not decrease the volume output or the alkalinity of the pancreatic juice in either group even though in the 'chronic' series 4 animals had gastric ulcers and one had a duodenal ulcer as seen at autopsy.

The mean control secretion of juice for the 'acute' dogs (table 3) was much smaller in volume than that of the 'chronic' dogs. Although the weights of the dogs are not eliminated as a factor, this difference suggests an increased sensitivity to secretin in the 'chronic' dogs. Furthermore, the mean increase in volume and alkali is much smaller in the 'chronic' dogs following an intravenous injection of sodium cinchophen than it is in the 'acute' dogs.

The presence of excess sodium bicarbonate in the sodium cinchophen solution may have accounted for the above results; however, intravenous administration of 5 per cent sodium bicarbonate in volumes equal to those of the sodium cinchophen administered, increased the volume and alkali content of the juice to a much smaller extent than did the sodium cinchophen in the acute experiments (table 4). Again, while the sodium cinchophen was being introduced N/10 hydrochloric acid was

TABLE 3. COMPARISON OF MEAN RESULTS BEFORE AND AFTER CINCHOPHEN IN 'ACUTE' AND 'CHRONIC' DOGS

VOLUME BEFORE CINCHOPHEN		ALKALI BEFORE CINCHOPHEN		VOLUME AFTER CINCHOPHEN		ALKALI AFTER CINCHOPHEN	
'Acute'	'Chronic'	'Acute'	'Chronic'	'Acute'	'Chronic'	'Acute'	'Chronic'
1.91	3.50	3.03	4.63	3.32	4.04	5.50	5.60

TABLE 4. RESULTS FOLLOWING ADMINISTRATION OF 5 PER CENT SODIUM BICARBONATE IN VOLUMES EQUAL TO THOSE OF CINCHOPHEN SOLUTION ADMINISTERED<sup>1</sup>

MEAN VOLUME BEFORE NaHCO <sub>3</sub>	MEAN ALKALI BEFORE NaHCO <sub>3</sub>	MEAN VOLUME AFTER NaHCO <sub>3</sub>	MEAN ALKALI AFTER NaHCO <sub>3</sub>	VOLUME	ALKALI
cc.	cc. N/10 NaOH	cc.	cc. N/10 NaOH		
2.36	2.37	3.11	3.27	+31.8	+38

<sup>1</sup> Each figure mean of three 15-minute periods.

injected into the other femoral vein in amounts which would much more than neutralize any free bicarbonate in the test sodium cinchophen solution. The volume and alkali content of the juice were increased as before.

#### DISCUSSION

The results presented above make it clear that cinchophen decreases neither the volume nor the alkali content of the external secretion of the pancreas. Apparently it increases both, and this despite the fact that in the dogs previously receiving 200 mg/kg. of cinchophen daily for 4 days peptic ulceration was present in the pyloric part of the stomach, and in one case in the first part of the duodenum. The ability of the pancreas to secrete fluid and alkali remained normal. This is in direct contrast to the effect of cinchophen upon the juice secreted from the Brunner's gland bearing part of the duodenum (1). Cinchophen peptic ulceration, in dogs, is much commoner in the pylorus than in the duodenum and perhaps here we have the reason; although the secretion from the Brunner's glands is decreased, that of the pancreas is increased or unchanged, and that from the liver, until parenchymatous

destruction occurs, is also increased. The pH of both these secretions is much higher than that of gastric juice; thus some degree of neutralization of the gastric acid may still take place and damage to the duodenal mucosa may be rendered less likely. These results may also explain why a Ramstedt operation may offer partial protection to dogs against cinchophen-induced ulcers (5), by allowing free regurgitation of pancreatic juice and bile into the stomach.

#### SUMMARY AND CONCLUSIONS

The intravenous administration of 100 mg/kg. of neutralized sodium cinchophen produced an increase in both the volume and alkali content of pancreatic juice from the dog's pancreas stimulated to secrete submaximally by continuous intravenous secretin infusion. The previous oral administration of cinchophen, to the point of ulcer production, for 4 days failed to alter the response of the pancreatic secretion to intravenous sodium cinchophen. The bearing of these results upon the occurrence of cinchophen peptic ulcer is discussed.

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# EFFECT OF SURGICAL EXTIRPATION OF PYLORIC PORTION OF THE STOMACH ON RESPONSE OF FUNDIC GLANDS TO HISTAMINE AND URECHOLINE IN DOGS

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**R**ECENTLY we reported (1) that irrigation of the mucosa of the pyloric portion of the stomach with a solution of acetylcholine caused strong stimulation of acid secretion by the fundic glands, apparently due to a release of gastrin from the pyloric mucosa.

The hypothesis that cholinergic stimuli release gastrin from the pyloric mucosa was further studied by determining the effect of surgical extirpation of an isolated pyloric pouch on the acid secretory response of a fundic pouch to subcutaneously administered Urecholine (urethane of beta methylcholine). As a control procedure the response of the fundic pouch to histamine was determined before and after pylorectomy. The response to histamine and Urecholine given simultaneously was also studied before and after pylorectomy.

## METHODS

Dogs with two gastric pouches, one pyloric and one fundic, were prepared by the method described in our earlier report (1). Gastric juice was collected only from the fundic pouch. The dogs were fasted for at least 12 hours before each test and collections were made for half an hour before injection of drugs to insure that the animals were in a basal state as indicated by absence of free acid from the juice. After injection of the drug being tested, collections of juice were continued for six 10-minute periods. Volume was recorded and free acid concentration determined by titration with 0.03 N NaOH using *p*-dimethylaminoazobenzene as indicator. Free acid output in millimoles was calculated by multiplying the volume in cubic centimeters of each sample by its acid concentration in normality. Histamine diphosphate was given in a dose of 0.07 mg/kg., Urecholine<sup>1</sup> in doses of 0.07 mg/kg. and 0.14 mg/kg. and histamine diphosphate and Urecholine together in a dose of 0.07 mg/kg. of each drug. All drugs were given subcutaneously. After the response to these drugs had been established by repeated testing in each dog the pyloric pouch was completely removed surgically and then the tests were repeated after a one-week post-operative period.

## RESULTS AND COMMENT

Table 1 summarizes the results. The free acid output in response to histamine was not significantly different before and after pylorectomy but the response to

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<sup>1</sup> Kindly supplied by Dr. R. C. Pogge, Merck and Co.

Urecholine fell markedly. With the lower dose of Urecholine the decrease was greater than 90 per cent in both animals; with the higher dose it was 80 per cent in one dog and 96 per cent in the other. It is clear that pylorectomy selectively influences the response of the fundic pouch to Urecholine.

The response to the two drugs given simultaneously was not significantly affected by pylorectomy. This would appear to be a puzzling result but is explainable, we believe, on the basis of the hypothesis that cholinergic effects although completely or relatively ineffective as direct stimuli to the parietal cell are capable of acting directly upon the parietal cell to potentiate the response to other stimuli (2). The present results would further indicate that when histamine and Urecholine are acting together in the doses we have used, this potentiating effect is the only im-

TABLE 1. VOLUME AND FREE ACID OUTPUT FROM FUNDIC POUCH IN RESPONSE TO HISTAMINE AND URECHOLINE BEFORE AND AFTER SURGICAL REMOVAL OF PYLORIC POUCH

DOG	DRUG	DOSE	BEFORE PYLORECTOMY			AFTER PYLORECTOMY		
			No. of Tests	Volume	Free Acid Output	No. of Tests	Volume	Free Acid Output
				cc/hr.	mm/hr.		cc/hr.	mm/hr.
A	Histamine <sup>1</sup>	0.07	4	11.6 ± 0.71	0.840 ± 0.084	10	8.5 ± 0.628	0.875 ± 0.093
	Urecholine	0.07	4	11.1 ± 1.01	1.052 ± 0.124	10	2.3 ± 0.154	0.112 ± 0.005
	Urecholine	0.14	2	28.7	5.49	3	11.8	1.065
	Histamine plus Urecholine	0.07 of each	5	35.0 ± 1.15	4.260 ± 0.648	10	28.2 ± 2.71	4.080 ± 0.45
B	Histamine	0.07	4	11.1 ± 1.02	2.143 ± 0.634	10	12.2 ± 1.25	1.690 ± 0.101
	Urecholine	0.07	4	11.8 ± 3.59	1.340 ± 0.475	10	0.1 ± 0.022	0.010 ± 0.004
	Urecholine	0.14	2	36.2	7.119	3	4.4	0.314
	Histamine plus Urecholine	0.07 of each	5	35.3 ± 4.09	5.670 ± 0.730	10	37.0 ± 3.86	5.306 ± 0.530

<sup>1</sup> Histamine diphosphate.

Values following means are standard errors.

portant one since the gastrin-releasing action from the pyloric mucosa has been eliminated.

After pylorectomy, although the response to Urecholine is greatly reduced it is not abolished. This residual activity could be due either *a*) to release of gastrin by Urecholine from some site other than the pyloric mucosa or *b*) to a direct action of Urecholine on the parietal cell. No choice can be made between these two possibilities at the present time because neither of them has been established as a physiological mechanism, i.e. there is no clear proof for the existence of gastrin in any tissue other than pyloric mucosa and there is likewise no proof that cholinergic stimuli can directly activate the parietal cell (1, 2). The results of the present study are thus in keeping with the hypotheses set forth in our earlier report (1), namely, that cholinergic stimuli *a*) release gastrin from the pyloric mucosa, and *b*) potentiate the response to stimuli acting directly upon the parietal cell.



## SUMMARY

In dogs with two gastric pouches, one pyloric and the other fundic, surgical removal of the pyloric pouch had the following effects on the acid secretory response of the fundic pouch to subcutaneously injected drugs: *a*) the response to histamine was not changed significantly, *b*) the response to Urecholine, a parasympathomimetic drug, was markedly reduced (over 80%) and *c*) the response to histamine and Urecholine given simultaneously was not significantly altered. These findings are in accord with the hypothesis that cholinergic stimuli *a*) release gastrin from the pyloric mucosa and *b*) potentiate the response of the parietal cell to other stimuli.

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## LIPIDS OF LYMPH FOLLOWING FEEDING OF FAT: AN EXPERIMENTAL STUDY

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THE observation made by Olof Rudbeck (1, 2) in 1653 that hepatic lymph always looked like water even when the intestinal lymph was very milky, as in the fed animal, is indicative of the marked differences in fat content of these two fluids. Quantitative studies of such differences are rare in the literature, probably owing to the difficulties involved in the collection of hepatic lymph, particularly. Large increases in the neutral fat content of lymph from the thoracic duct have been found by Zawilski (3) and many others following feeding of fat to dogs. Simultaneous increases were found in the phospholipid and cholesterol content of thoracic duct lymph of dogs (4), or intestinal lymph of rabbits (5, 6), fed fat prior to the operation necessary for the collection of the lymph. When the fat was fed after operation to the anesthetized dog, no change was found in the phospholipid content of the thoracic duct lymph (7).

We have measured the changes in composition of fat which occur in the lymph following feeding of a fat meal to dogs with fistulas of either the thoracic duct or hepatic lymphatics. In other acute experiments, a fat meal was given a few hours prior to operation, and then lymph was collected simultaneously from the hepatic and intestinal lymphatics for analysis. When hepatic lymph was collected without contamination by intestinal lymph the concentration of fat in this was always found to be low and similar to that of plasma even when the concentration in the intestinal lymph was greatly elevated after a fat meal.

### METHODS

For the collection of lymph from the thoracic duct, liver or small intestine a cannula of flexible polyvinyl tubing was introduced into the thoracic duct near its origin in the cisternum chyli, hepatic lymphatic trunk or main mesenteric lymphatic trunk of the dog as previously described (8). The lymph was collected continuously in heparinized tubes. At intervals blood was taken for comparison.

The lymph or plasma was extracted with Bloor's alcohol-ether mixture for an hour at room temperature. Aliquots were taken for the determination of cholesterol by the method of Bloor, Pelkan and Allen (9), and of phospholipid phosphorus by the method of Fiske and SubbaRow (10) after preliminary ashing with sulfuric acid and superoxol. Total lipids were determined gravimetrically after re-extraction with petroleum ether, or total fatty acids were determined by the method of Hunter, Knouff and Brown (11).

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## RESULTS

*Thoracic Duct Lymph.* Thoracic duct fistulas were established in 11 mongrel dogs, which weighed from 12.6 to 20.4 kg., after they had been fasted 40 to 48 hours. Under these circumstances the lymph was faintly cloudy. The mean concentration of total lipids in the sample of lymph taken just before the first meal after operation in 9 animals was 626 mg/100 ml. with a standard error of 35. When a fat-free meal

TABLE 1. EFFECT OF FEEDING ON TOTAL LIPIDS IN THORACIC DUCT LYMPH

ANIMAL	FAT FED <sup>1</sup>	DAYS AFTER OPERA- TION	TOTAL LIPIDS, MG/100 ML.									
			Hours After Meal									
			0	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-9	9-12
<i>First meal after operation</i>												
1	Oleic acid	1	553	1870	2080	2050	3480	4440	4440			
2	Oleic acid	1	620	833	4350	4350	7890	7890	7890			
3	Oleic acid	1	700	2670	880	880	2080	2080	2080			
4	Butter	0.25	460	437	3800	3800	7340	7340	7340	3140	3140	3140
5	Butter	0.21	567	950	3040	3040	2420	2420	2420	2430	2430	
6	Butter	0.29	840	1620	4490	4490	5160	5160	5160	4710	4710	4710
7	Cream and eggs	1	600	1150	2600	2600	3230	3230	3230			
8	Cream and eggs	2	673	517	580	1750	1750	1750	2830	2830	2830	2830
9	Crisco	0.23	621	1340	3960	4670	4090	3210	1550	2030		
10 <sup>2</sup>	None	1	737	787	787	521	521	421	421	449	449	449
11 <sup>2</sup>	None	1	1042	743	743	721	721					
<i>Subsequent meals</i>												
1	Butter	4	2150	2300		4060			6280			
4	Cod liver oil	1.67	503	530	1810	1810	3400	3400	3400			
6	Butter	4	650	973	2190	2190	3060	3060	3060	2780	2780	2780
9	Oleic acid	1	5710	6950	5710	6420	5470	5220	5620	2920		
9	None	2	1330	2050	2960	1500	2100	1740	1570			
9	Crisco	5	3970	4100	7020	5990	5030	3180	2410	3560		

<sup>1</sup> The basic fat-free meal consisted of 30 gm. casein, 84 gm. cornstarch and 6 gm. salt mixture. To this basic meal 30 gm. of fat was added either as free oleic acid plus an equivalent amount of glycerin, or as butter, Crisco, or cod liver oil, or a meal consisting simply of 200 ml. thick cream (36% fat) and 4 egg yolks was given.

<sup>2</sup> Values given are for total fatty acids.

was given no increase in total fatty acids of lymph was found. If fat was given with the meal, the lymph usually became very milky within the first hour, and the concentration of total lipids increased to very high levels within 6 hours (table 1). This was true whether the fat administered was the free fatty acid, oleic acid, or the triglycerides contained in butter, cream, Crisco, or cod liver oil. In all cases large quantities of milky fat were absorbed by way of the lacteals. Freeman and Friedemann (12) have also found a large increase in the concentration of neutral

fat of thoracic duct lymph after feeding oleic acid to dogs. Frazer (13), however, postulated two routes for the absorption of fat; one the lacteals for the triglycerides with another route for the free fatty acids.

The process by which the concentration of lipids increases in thoracic duct lymph is not simple but involves many steps including the emptying of the stomach, emulsification and enzymatic hydrolysis of the fat in the intestine, absorption and resynthesis in the mucosal cells, release of the fat from the cells, and propulsion of it through the lacteals. It is not surprising, then, that large variations were found in the level of total lipids at each time interval after feeding in these dogs. Although the animals were able to absorb large amounts of fat even rather early after operation, it is probable that the rate of fat absorption is even higher in normal dogs. Indeed, 18 and 24 hours after the first meal the concentration of total lipids had not returned to normal after 4 of 6 meals studied. Also when the fat-free meal was given to animal 9, 24 hours after the oleic acid meal, an increase in total lipids was found which would suggest that some of the oleic acid had remained in the lumen of the

TABLE 2. PERCENTAGE COMPOSITION OF LIPIDS IN THE THORACIC DUCT LYMPH

TOTAL LIPIDS, MG/ 100 ML.	NEUTRAL FAT <sup>1</sup>			PHOSPHOLIPID			CHOLESTEROL		
	Samples, No.	% <sup>2</sup>	S.D.	Samples, No.	% <sup>2</sup>	S.D.	Samples, No.	% <sup>2</sup>	S.D.
437-1000	15	51.3	9.0	19	30.5	5.8	15	18.1	3.4
1001-2000	11	71.5	6.1	13	19.9	5.0	11	8.1	1.5
2001-3000	17	79.5	4.5	13	14.9	3.4	1	5.7	1.5
3001-4000	9	84.1	1.6	20	12.0	1.3	9	3.7	0.9
4001-8000	22	85.9	2.2	24	11.1	1.8	22	2.8	1.9

<sup>1</sup> Calculated by subtracting the sum of values for phospholipid and cholesterol from that of total lipid.

<sup>2</sup> Percentage of total lipids.

gastro-intestinal tract for more than 24 hours. Earlier studies of the lipid levels of blood and lymph would indicate that fat absorption from similar meals is complete in less than 24 hours in the normal dog.

All of the samples of lymph from the thoracic duct of the 9 animals which were fed the fat meals have been arranged in groups according to the level of concentration of total lipids. The proportion of the total lipids which was neutral fat increased progressively from 51.3 per cent to 85.9 per cent as the concentration of fat in the lymph increased (table 2). Associated with this great increase in the triglycerides there was a decrease in percentage of phospholipid from 30.5 per cent to 11.1 per cent and of cholesterol from 18.1 per cent to 2.8 per cent. In spite of this percentage decrease in phospholipid, there was an actual twofold or threefold increase in the concentration of the phospholipid (table 3). The increases in cholesterol were much smaller and of questionable significance.

**Hepatic Lymph.** Hepatic lymphatic fistulas were established in 5 dogs, of 16 to 21.8 kg. body weight, which had been fasted from 19 to 72 hours before operation. Any cross-channels from the intestinal lymphatics which were visible at operation

were ligated. Clear lymph was collected continuously for 8 to 24 hours, and then a fat meal was fed. In 2 of these animals the lymph remained fairly clear for the 8 hours studied after the meal, and the increases found in the total lipids were relatively small (table 4). In the other 3 animals the lymph became very milky and the concentration of total lipids increased as markedly as that of thoracic duct lymph after a similar meal. To determine whether the fat content of the hepatic lymph had actually increased or whether intestinal lymph was flowing through the hepatic lymphatic fistula, an exploratory operation was performed. Water-clear lymph was observed in the hepatic lymphatic ducts above the fistula and mixing of milky intestinal lymph with this clear lymph was easily seen at the site of insertion of the plastic cannula. Some cross-channels between the hepatic and mesenteric lymphatics not visible at the time of operation must have opened up with the increased flow of intestinal lymph which follows feeding. These unsuccessful experiments illustrate the difficulties in getting hepatic lymph uncontaminated by the adjacent intestinal lymphatics in the dog.

TABLE 3. CONCENTRATION OF LIPIDS IN THE THORACIC DUCT LYMPH

NEUTRAL FAT <sup>1</sup>		PHOSPHOLIPID <sup>1</sup>		CHOLESTEROL <sup>1</sup>	
mg/100 ml.	S.D.	mg/100 ml.	S.D.	mg/100 ml.	S.D.
360	140	197	43	119	21
1120	144	310	92	128	27
2010	304	351	117	143	39
2930	312	414	67	128	33
4800	1024	604	160	154	34

<sup>1</sup> Mean values for groups in table 1.

*Hepatic and Intestinal Lymph. Acute Experiments.* Fourteen dogs weighing from 6.5 to 24.0 kg. were fed a high fat meal containing 50 gm. each of Crisco and horse meat. Six hours later the lymphatics were observed and cannulated with the animal under ether anesthesia. In each animal at operation the intestinal lymphatics were observed to be distended with very white milky chyle, while the hepatic lymphatics were filled with a clear fluid sometimes slightly yellowish. In certain cross-channels mixing of the milky fluid coming from the intestine with the clear lymph coming from the liver was clearly visible. The lymphatics containing the milky fluid were ligated so that the clear lymph from the liver was not contaminated. Successful cannulations were made of the hepatic lymphatics in 2 animals, of the intestinal lymphatics in 4, and of both in 2. Lymph was collected for about one hour and analyzed.

The concentration of neutral fat in this hepatic lymph was similar to that of plasma, while the concentration in intestinal lymph was much higher (table 5). The concentration of phospholipid was often higher than that in the plasma while differences in cholesterol were not very significant. Unfortunately the anesthesia and the manipulation of the intestine during the operative procedure tend to inhibit the absorption of fat, as shown by the fact that the lymph in lacteals became

less opaque as the operation progressed. This no doubt accounts for the fact that the concentration of fat found in the intestinal lymph in these acute experiments is not so great as that in the lymph from the thoracic duct in table 1.

## DISCUSSION

The observation that the lymph of the small intestine and therefore of the thoracic duct becomes very milky after a meal, while lymph from the liver remains water-clear, confirms the very old observation of Olof Rudbeck. The mean concentration of fatty acids in the clear hepatic lymph of 6 dogs was 449 mg/ml., which was about the same as the concentration in the plasma of these animals. Since fatty acids make up approximately two thirds of the phospholipid molecule and are also esterified with approximately 70 per cent of the cholesterol, it can be calculated roughly from the data in table 5 that about 28 per cent of the total fatty acids of the hepatic lymph was present in the form of neutral fat. Thus the concentration of neutral fat in hepatic lymph is definitely lower than that in the

TABLE 4. TOTAL LIPIDS OF LYMPH FROM THE HEPATIC LYMPHATICS AFTER FEEDING

ANIMAL	FAT FED	DAYS AFTER OPERATION	TOTAL LIPIDS, MG/100 ML.								
			0	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8
1	Crisco	0.5	420	463	897	550	428	378	378	378	378
2 <sup>1</sup>	Crisco	0.35		312	312	312	639			818	
3	Crisco	1	750	1490	3320	3320	3320	3320	3320	3320	
4	Crisco	1	1120	1160	1570	1570	5180	518c	5180	5370	5370
5	Cream and eggs	0.33			562	1260	3340				

<sup>1</sup> Values given are for total fatty acids.

lymph of the small intestine or thoracic duct of fasting dogs. Increases in concentration of neutral fat from about 0.5 to 7 per cent were found in the lymph from the thoracic duct after feeding of a fat meal to dogs. Somewhat lower concentrations were found when intestinal lymph was collected with the animals under anesthesia. Whereas the major increase of lipids in lymph of the small intestine or the thoracic duct after a fat meal had been fed was in neutral fat, smaller increases, up to three-fold, did occur in the phospholipids as previously noted in intestinal lymph of the rabbit by Süllmann and Wilbrandt (5). Our evidence indicates that the output of neutral fat or phospholipid in the lymph from the liver is not changed during the absorption of fat from the intestine.

Since the concentration of phospholipid but not of cholesterol does increase appreciably in intestinal lymph during absorption of fat it is probable that the mucosa of the small intestine is a source for plasma phospholipids as well as neutral fat via the lymphatic system, during the absorption of fat. Fischler, Entenman, Montgomery and Chaikoff (14) have shown that the liver is the chief, if not the only, source of plasma phospholipids in dogs, but their animals were not studied during the absorption of fat.

## SUMMARY

A study has been made of the concentration of lipids in lymph from the thoracic duct, small intestine and liver of the dog before and after feeding. The concentration of total lipids or total fatty acids of lymph from the small intestine or thoracic duct increased enormously after the administration of a meal rich in triglycerides or the free fatty acid, oleic acid, owing largely to an increase in concentration of neutral fat. A threefold increase in concentration of phospholipid was associated with the maximal increases found in neutral fat. Little if any change was found in the cholesterol content of the lymph. The finding of a definite increase

TABLE 5. CONCENTRATION OF LIPIDS IN HEPATIC LYMPH,  
INTESTINAL LYMPH AND PLASMA

ANIMAL	TIME AFTER FEEDING	TOTAL FATTY ACIDS <sup>1</sup>			PHOSPHOLIPIDS <sup>1</sup>			CHOLESTEROL <sup>1</sup>		
		He- patic Lymph	Plasma	Intestinal Lymph	He- patic Lymph	Plasma	Intestinal Lymph	He- patic Lymph	Plasma	Intestinal Lymph
	hours									
1	3		360	1160		382	298		217	85
2 <sup>a</sup>	3		258	1610		232	260		125	60
3 <sup>2</sup>	3	477	344		330	360		106	156	
4 <sup>2</sup>	3-4	391	466		338	472				
5 <sup>2</sup>	4	424	446		495	470		186	254	
6	6	278	333		274	290				
7	6	515	555	1240	408	299	156		166	
8	6	610	416	2700	430	312	332	165	160	135
9	6		460	1150		438	210			
10	6		457	2440		340	268			
11	6		484	3720		370	228		232	183
12	9½			2350			521			170
12	12		405	1660		378	299			143
12	15			3710			526			121

<sup>1</sup> Milligrams per 100 ml.

<sup>2</sup> These animals received 50 cc. of cream only. The other animals received larger meals containing 30 to 50 gm. of Crisco or simply 250 to 500 cc. of cream.

in phospholipid concentration of intestinal and thoracic duct lymph suggests that the mucosa of the small intestine is normally a source of phospholipids for the plasma during the absorption of fat. The concentration of total fatty acids in the hepatic lymph was lower than that of intestinal or thoracic duct lymph and did not increase after feeding. The concentration of phospholipids and cholesterol of hepatic lymph was also similar to that of plasma and did not change appreciably during the absorption of fat from the intestine.

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# EFFECT OF DIETARY SUBSTANCES ON CHOLATE SYNTHESIS IN THE DOG

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**A**LTHOUGH there is considerable evidence that cholate output in dogs' bile increases with the amount of mixed diet that is eaten (1-4), the specific dietary material(s) necessary for cholate synthesis have not been identified.

For example, the effect of addition of pure carbohydrate to a control regime has been reported on only one dog (1). Addition of various fats to various control diets has produced inconsistent changes in cholate output in 5 dogs (3, 5, 6). Cholesterol or cholesterol-rich foods given orally (3, 7) or cholesterol intravenously (8, 9) has also produced inconsistent changes in cholate output, although deuterium-cholate has been recovered following injection of deuterium-cholesterol in a bile fistula dog (10), suggesting biological conversion of cholesterol to cholate.

Although cholate output has been roughly proportional to the protein fed in mixed diets (1-4), 24-hour cholate outputs have not been significantly altered by addition of casein hydrolysate or 10 pure amino acids (11), by individual amino acids (5, 12-14), yeast (3), liver extract (11) or vitamin mixtures (11) to various control regimes in tests on 4 or fewer dogs.

Accordingly, the present study was undertaken in order further to identify dietary substances possessing cholepoietic activity.

## METHODS

Two types of bile fistula dogs were used. Cholecystonephrostomized (internal bile fistula) dogs were prepared (15) so that all bile could be collected with the urine. Since no bile reached the intestine, the cholate in the urine represented the amount synthesized. Cholic acid was measured by means of a spectrophotometer after developing color with furfural and  $H_2SO_4$  (16) in 5-day urine collection. Application of this method to urine was validated by demonstration that urine of 5 normal dogs was free of cholate and that added cholate was recovered quantitatively from the urine of 5 normal and 5 cholecystonephrostomized dogs. Tests using internal fistula dogs consisted of 2 or more consecutive 6-day periods. After the first of these, one or more levels of a test substance was added to the regime given during the first period, which was either a mixed (commercial dog food) or a synthetic (sucrose or gelatin plus casein hydrolysate) diet. Urine was discarded the first day of each period.

The second type of dog that was used was a modified Rous-McMaster external bile fistula (17) in which cholic acid was measured (16) in the 24-hour bile output.

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In addition, the dog was prepared with an indwelling duodenal catheter for administration of fluid materials. Tests using external fistula dogs consisted of 3-day periods. Only one test was performed per week on a dog. Thus, dogs received a mixed diet for 3 days per week, were completely fasted for one day, and were given 500 cc. of one per cent NaCl or NaCl plus a test material, given in 10 divided doses at 30-minute intervals, on each of the remaining days.

TABLE 1. TWENTY-FOUR-HOUR CHOLATE YIELDS FROM SUBSTANCES ADDED TO BASAL REGIMES IN INTERNAL BILE FISTULA DOGS

TEST SUBSTANCE	AMOUNTS ADDED	NO. DOGS	NO. TESTS	<i>a</i>	CHOLATE YIELDS/GM. OF TEST SUBSTANCE <i>b</i> ± <i>sb</i>
	gm/day			mg/day	
Series 1. Basal regime = 58 gm. (dry wt.) mixed diet/day (350 cal.)					
Mixed diet.....	58-174	6	23	612	+4.31 ± 0.45 <sup>2</sup>
Lard.....	10-36	16	34	847	+4.80 ± 3.0
Sucrose.....	25 and 50	4	12	953	-5.24 ± 4.55
Commercial casein.....	25 and 50	9	27	820	+25.1 ± 3.42 <sup>2</sup>
Zein.....	25 and 50	5	15	610	+20.8 ± 3.8 <sup>2</sup>
Gelatin.....	25 and 50	6	18	1067	+1.67 ± 4.68
Gelatin plus DL-tryptophane...	25 and 50	2	6	860	+1.50 ± 1.12
	25 and 50				
'Vitamin-free' casein.....	25 and 50	7	21	912	+6.48 ± 2.05 <sup>2</sup>
Casein hydrolysate.....	25 and 50	10	30	831	+2.12 ± 1.94
Brewer's yeast.....	25 and 50	4	12	868	+5.65 ± 2.42
Yeast plus gelatin.....	5 and 10	5	15	710	+3.20 ± 1.12 <sup>1</sup>
	25 and 50				
Yeast plus casein hydrolysate...	5 and 10	6	18	721	+5.17 ± 1.71 <sup>1</sup>
	25 and 50				
Yeast plus sucrose.....	5 and 10	5	10	816	+1.80 ± 4.8
	25 and 50				
Series 2a. Basal regime = sucrose-synthetic diet, 800 cal/day					
Commercial casein.....	25 and 50	7	20	599	-0.6 ± 1.7
Brewer's yeast.....	15 and 30	5	15	660	+4.4 ± 3.9
Liver concentrate.....	0.6-2.4	6	18	750	+21.8 ± 67
Series 2b. Basal regime = gelatin-synthetic diet, 800 cal/day					
Commercial casein.....	50	3	6		+9.2 <sup>3</sup> ± 1.04 <sup>2</sup>

<sup>1</sup> Significant at 5 per cent probability. <sup>2</sup> Significant at one per cent probability. <sup>3</sup> Calculated from mean increase in cholate output.

With both types of dogs the order in which different substances were tested was systematically varied among animals to control possible effects of time and of the preceding regime on cholate synthesis.

## RESULTS

With all substances which increased cholate output, the relationship between cholate output and the quantity of substance fed appeared to be linear when graphed.

Furthermore, covariance analysis (18) revealed that regression coefficients obtained from different dogs while on a given regime differed in random fashion. Accordingly, an average regression coefficient obtained by covariance analysis was used as a direct measure of the cholate yield, expressed in units of mg. cholate per gm. of test substance added to the basal regime. Thus, from the  $a$  constants and yields shown in table 1 the average daily cholate output may be calculated over the specified ranges of test-food intakes, according to the expression cholate output per day =  $a + b$  times test food intake per day. The significance of each yield,  $b$ , was obtained by dividing by the indicated standard error,  $sb$ , in the table.

The data in table 1 show that feeding the mixed diet (Pard, Swift and Company) significantly increased the daily output of cholate. Expressed as a yield, the increase was 4.3 mg. cholate per gm. of mixed diet. Neither lard nor sucrose had statistically significant effects on cholate synthesis. However, further tests (not shown in table) wherein 200 gm. of sucrose were added to 58 gm. of mixed diet in 5 dogs and to 116 gm. of mixed diet in 6 dogs showed a small ( $-2.3$  mg./gm. sucrose) but significant (paired comparison) decrease in cholate output.

Since the above findings suggested that neither fat nor carbohydrate could account for the cholepoietic action of the mixed diet, the yields from 3 commercial (non-purified) proteins were determined. Casein and zein gave high yields whereas gelatin was without effect. Further experiments summarized in table 1 indicate that the cholepoietic actions of casein, zein, and the mixed diet are not entirely dependent upon amino acids, since  $a$ ) the supplementation of gelatin with tryptophane did not induce cholepoiesis, contrary to an early claim (13),  $b$ ) vitamin-free casein (Lab-Co) gave a significantly smaller yield of only 26 per cent of that obtained from commercial casein, and  $c$ ) enzymatic casein hydrolysates (Amigen and Protolysate, Mead Johnson & Company) which presumably have the same amino acid composition as casein were completely inactive.

Since the above results suggested that a vitamin may be concerned in cholepoiesis, Brewer's yeast was tested. Yeast alone was ineffective, but small amounts added to gelatin or to casein hydrolysate produced small but significant cholate yields from the mixtures. However, since further covariance analysis revealed no significant differences between yields from gelatin or hydrolysate alone and each plus yeast, Brewer's yeast cannot be considered a proven source of cholepoietic material. The small yields from the mixtures may be due to additive effects of traces of cholepoietic material(s) in yeast and in the proteins.

In all preceding experiments, test substances were added to an inadequate calorie diet containing cholepoietic material. Since it seemed desirable to assay substances for cholepoietic activity without possible complications from such a basal regime, a synthetic diet consisting of 150 gm. of sucrose, 50 gm. of Protolysate, 25 gm. of lard, and 5 gm. of NaCl was prepared. When fed to 6 dogs for 3 successive 6-day periods, this diet did not depress cholate output as compared with fasting outputs obtained during 5-day tests in 2 of the same and 3 additional dogs. This means that dogs can maintain cholate synthesis (about 700 mg./day) for periods of at least 18 days when the diet is composed of substances with little or no cholepoietic potency as judged by results shown in series 1, table 1.

However, neither commercial casein, Brewer's yeast, nor liver concentrate increased cholate output when added to the sucrose-synthetic diet, as shown in *series 2a*, table 1. On the assumption that sucrose interfered with cholepoiesis from commercial casein, a second synthetic diet was prepared in which gelatin replaced sucrose. When commercial casein was again tested (*series 2b*, table 1), a significant yield was obtained, though considerably less than the yield from commercial casein when added to a mixed diet as in *series 1*, table 1. These results revealed a synergism between commercial casein and the mixed diet, since the cholate output when both were present was greater than could be accounted for by their additive effects. This finding suggested the existence of at least 2 separate cholepoietic factors.

In the next series of experiments (table 2), materials were given intraduodenally to external fistula dogs, and cholate outputs were compared with those obtained when only saline was given. Under these conditions, commercial casein gave a cholate

TABLE 2. TWENTY-FOUR-HOUR CHOLATE OUTPUTS IN EXTERNAL BILE FISTULA DOGS

TEST SUBSTANCE	AMOUNTS ADDED	NO. OF DOGS	CHOLATE OUTPUTS			CHOLATE YIELDS TEST SUBSTANCE <sup>3</sup>
			Saline Control	Test	$\mu$ Ratio	
	gm/day		gm/day			mg/gm
Commercial casein.....	50	7	361	734	4.70 <sup>3</sup>	7.5
Casein hydrolysate.....	50	5	325	498	2.17	inactive
Vitamin mixture.....	1 capsule	6	385	396	0.18	inactive
Sucrose.....	50	6	385	407	0.33	inactive
Liver concentrate.....	2.5	7	361	466	3.06 <sup>4</sup>	42.0
Hydrolysate plus vitamin mixture.....	50 1 capsule	5	325	416	0.94	inactive
Hydrolysate plus liver concentrate.....	50 2.5					
		6	319	708	11.75 <sup>5</sup>	7.8 <sup>5</sup> 155 <sup>6</sup>

<sup>1</sup> From paired comparison analysis. <sup>2</sup> Calculated only for active test substances. <sup>3</sup> Significant at one per cent probability. <sup>4</sup> Significant at 5 per cent probability. <sup>5</sup> Yield from hydrolysate assuming liver concentrate to be inactive. <sup>6</sup> Yield from liver concentrate assuming hydrolysate to be inactive.

yield of 7.5 mg/gm., essentially the same as when added to a gelatin-synthetic diet. Casein hydrolysate was again without significant effect, as was a mixture of synthetic vitamins, the composition of which is given in table 3, and as was sucrose. However, liver concentrate (partial vitamin composition shown in table 3) produced a cholate yield of 42 mg/gm., the highest yet found.

In order to obtain further evidence concerning operation of 2 or more cholepoietic materials, casein hydrolysate was supplemented with the vitamin mixture and with liver concentrate, respectively. The former combination was without significant effect, but casein hydrolysate plus liver concentrate produced yields which may be calculated as 7.8 mg/gm. of hydrolysate or 155 mg/gm. of liver concentrate, assuming that the response is due to one or the other substance. These results suggest that liver concentrate restored the activity of hydrolysate to that of commercial casein. The synergism between hydrolysate and liver concentrate furnishes further evidence for the existence of at least 2 cholepoietic factors.

## DISCUSSION

The present findings confirm previous reports that cholate synthesis is increased by feeding a mixed diet (1-4), and demonstrate a linear relationship between cholate output and the quantity of cholepoietic material fed. Such a relationship provides a valid basis for calculating the yield of cholate per gm. of substance fed within a specified range.

Sucrose was found to have unique effects on cholepoiesis, not altering endogenous, or fasting, cholate synthesis as shown by its inactivity when compared with saline control outputs. However, sucrose inhibited the action of exogenous cholepoietic substances as shown by the decreased output of cholate when sucrose was added to mixed diets, and by the apparently complete suppression of the cholepoietic action of commercial casein when casein was added to a sucrose-synthetic diet.

The cholepoietic action of crude proteins such as commercial casein was shown not to depend entirely upon amino acids, since either purification by hot alcohol extraction or enzymatic hydrolysis reduced or eliminated it. These findings are in

TABLE 3. COMPOSITION OF LIVER CONCENTRATE AND VITAMIN MIXTURE

	SYNTHETIC VIT. A	ACTIVATED ERGOS- TEROL	THIAMINE	RIBOFLAVIN	NIACIN	PANTOTHENIC ACID	PYRIDOXINE	ASCORBIC ACID	DL-TOCOPHEROL	CHOLINE	FOLIC ACID	B <sub>12</sub>	NITROGEN
	USP units	USP units	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	%
Liver concentrate/ gm. <sup>1</sup> .....	?	?	?	.25	1.5	.5	.04	?	?	15	.012	.0025	6.5
'Supradin'/capsule <sup>2</sup> ...	25,000	1000	10	5.0	50.0	10	3.0	150	3.0	0	0	0	?

<sup>1</sup> Material and analysis furnished by Wilson Laboratories.

<sup>2</sup> Material and analysis furnished by Hoffmann-La Roche.

accord with a recent failure to demonstrate clear-cut evidence of cholepoiesis from amino acid mixtures or casein hydrolysate (11).

The existence of a vitamin-like substance which is concerned in cholepoiesis is supported by the high cholate yields obtained from liver concentrate. This substance is not contained in Brewer's yeast in appreciable quantities, nor is it present in the synthetic vitamin mixture which was tested. A comparison of the vitamin composition of liver concentrate with that of the inactive vitamin mixture suggests that choline, vitamin B<sub>12</sub>, or pteroylglutamic acid may be the active factor. These substances are now being tested.

The synergistic actions of commercial casein and the mixed diet and of liver concentrate and casein hydrolysate establish the existence of at least 2 cholepoietic agents. One of these is presumably the unidentified 'vitamin,' and the other is presumably some amino acid(s). It appears that the vitamin is present in the mixed diet, commercial casein and zein, and in liver concentrate, but not in gelatin, purified or hydrolysed casein, Brewer's yeast, or the vitamin mixture. The amino acid(s) appear to be present in the mixed diet, proteins, and to a small extent only in liver

concentrate, but not in yeast or the vitamin mixture. It further appears that during fasting the factor limiting cholepoiesis is the vitamin, since liver concentrate but not casein hydrolysate is effective. However, when an excess of the vitamin is provided, as when liver concentrate is given, the supply of amino acid(s) limits cholepoiesis, as indicated by the increased cholate yield when hydrolysate is given together with liver concentrate. Thus, it now seems feasible to supply sufficient amino acid(s) and assay dietary substances for the vitamin, and to supply adequate vitamin and assay substances for the amino acid(s).

#### SUMMARY

The cholepoietic activity of various substances was determined quantitatively in biliary fistula dogs by feeding them alone or as supplements to mixed or synthetic diets. Lard had no effect on cholate synthesis, and sucrose inhibited cholate synthesis from exogenous, but not from endogenous factors. Liver concentrate showed the greatest cholepoietic activity of all substances tested, yielding 42 mg. of cholate per gm. of concentrate fed. Commercial casein gave a cholate yield of about 8 mg./gm., and a mixed dog food gave a yield of 4 mg./gm. Crude zein was about as potent as commercial casein, but 'vitamin-free' casein was less effective, and casein hydrolysate, gelatin alone or supplemented with tryptophane, Brewer's yeast, and a synthetic vitamin mixture were without effect. A synergistic effect on cholepoiesis was obtained by combining commercial casein with a mixed diet, and casein hydrolysate with liver concentrate. These findings of synergism suggest that at least 2 cholepoietic factors are concerned. One appears to be a 'vitamin' present in liver concentrate, commercial casein, zein, and the mixed diet, but not in yeast, the synthetic vitamin mixture, gelatin, or casein hydrolysate. The second factor appears to be an amino acid(s), present in certain proteins and in liver concentrate.

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# INVESTIGATION OF HEPATIC FUNCTION BY CLEARANCE TECHNIQUES<sup>1</sup>

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THE purpose of this investigation is to adapt the methods and concepts of renal clearance (1) to the study of hepatic function. The clearance of a given compound is usually defined as the virtual volume of blood (or plasma) completely cleared of that compound within a standard period of time. Clearance may be more realistically defined as the smallest volume of plasma at a given concentration which could have yielded the amount of material removed from the plasma. Mathematically, clearance may be stated as follows:

$$C \times P = Q_e,$$

where  $C$  = clearance,  $P$  = plasma concentration, and  $Q_e$  = the amount of material cleared either by excretion or metabolic conversion.

In studying renal function, the amount of material excreted,  $Q_e$ , is measured in the urine. However, in the study of hepatic function, a comparable collection of bile is useless because of the latent period between removal from plasma and appearance in bile (2). Furthermore, many compounds are cleared by storage or metabolic conversion. A technique for the measurement of hepatic clearance must then depend on some scheme to determine  $Q_e$  indirectly.

Our measurements of  $Q_e$  are obtained by utilizing an intravenous infusion at a constant rate. During the early part of the infusion, the plasma concentration rises rapidly and eventually reaches a stable equilibrium level. At this level the rate of excretion or removal is equal to the rate of infusion,  $R$ , in mg/minute. Earle and Berliner (3) have used this method successfully in measuring renal clearance where it was possible to compare  $R$  directly with  $Q_e$ .

If clearance is independent of plasma level in the range of values studied,  $P$  will be a simple linear function of  $R$ , and extrapolation of the line can pass either through the ordinate, the abscissa or the origin. If the line passes through the ordinate, the corresponding value of  $P$  will represent the threshold. If the line passes through the abscissa, the corresponding value of  $R$  will represent the maximum rate at which the liver can *completely* clear the blood during a single circulatory passage. If the line passes through the origin there is neither a threshold nor a level of complete clearance.

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In clinical studies, an empirical approximation of clearance is obtained by so-called tolerance and retention tests. In a previous paper (4) we discussed the relationship of these methods to clearance, and derived the following relationship:

$$\frac{C}{V} = \frac{(P - P')}{tPm}$$

where  $V$  = the volume of the fluid space containing the injected material,  $t$  = time elapsed between samples,  $P$  and  $P'$  the concentration of the initial and final plasma samples, and  $Pm$  = the mean plasma concentration during the time interval  $t$ . For practical clinical purposes,  $Pm$  may be taken as the geometric mean of  $P$  and

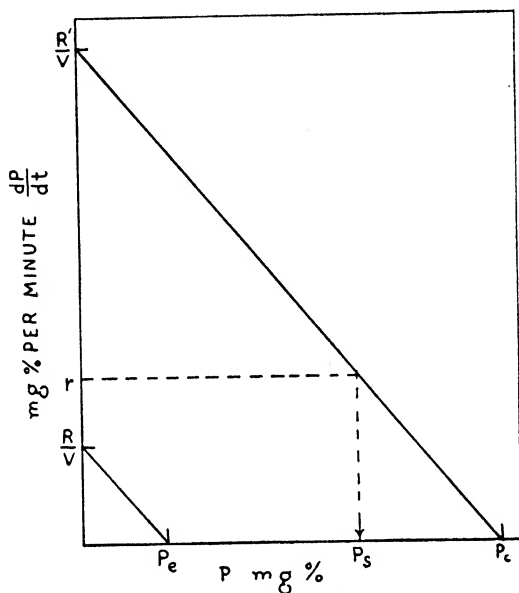


Fig. 1. PROPORTIONAL INCREMENT METHOD

$P'$ , which equals  $\sqrt{P \times P'}$ , but for the precision required in a theoretical analysis, it is necessary to consider the true mean. The relationship is then expressed as:

$$\frac{C}{V} \int_0^t P' dt = P - P',$$

which can finally be resolved as:

$$\frac{C}{V} = \frac{2.3 (\log P - \log P')}{t}$$

Although this last equation has been derived to apply to falling plasma levels after a single injection, calculations of  $C/V$  from the falling plasma levels after a period of constant infusion are more accurate, since possible errors due to mixing



time are eliminated. Furthermore, auxiliary removal systems (e.g. the reticulo-endothelial system), when present would be saturated and less likely to influence significantly the rate of fall in plasma levels.

The fractional clearance,  $C/V$ , may also be calculated from the rising plasma

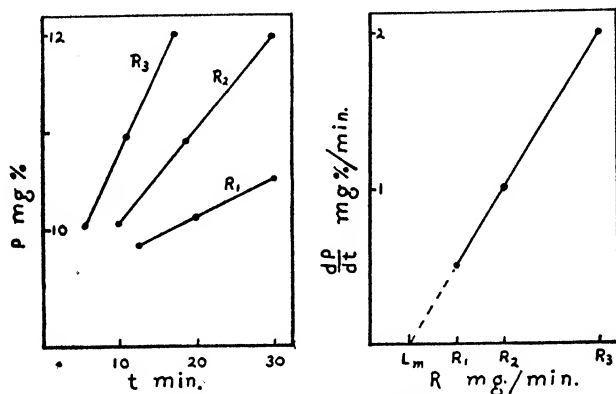


Fig. 2. SERIES INCREMENT METHOD

levels measured at the beginning of a constant infusion as indicated by the following derivation:

$$\begin{aligned}\frac{dP}{dt} &= \frac{R - CP}{V} \\ R &= CPe \\ \frac{dP}{dt} &= \frac{C(Pe - P)}{V} \\ \frac{C}{V} &= \frac{2.3 [\log Pe - \log (Pe - P)]}{t}\end{aligned}$$

where  $Pe$  = the plasma level at equilibrium. If some auxiliary clearance system, the activity of which is not a function of the plasma concentration, is present, the fractional clearances determined from rising plasma levels will differ significantly from the falling level fractional clearances. In the derivations above, it has been assumed that the injected material (bromsulfalein in this instance) is confined to the plasma. If 2 or more fluid compartments are entered to any significant degree at different rates, a graph of  $\log P$  plotted as a function of time would give a curve that was convex downward rather than a straight line. Fractional clearances calculated from points on the early portion of this curve would then differ from those calculated from later points.

In a quantitative study of the function of any organ, a distinction must be made between capacity and intensity. The intensity of an organ's function is defined as the rate at which it does some form of physiological work. The *maximum* rate at which the organ can function defines capacity. If the function of some organ changes in intensity while the capacity is unchanged, we may assume that the change is part

of an integrated response by the entire organism. If the capacity of the organ changes with or without a change in intensity, we can assume that the organ in question has been directly affected. Changes in capacity and intensity may be closely associated, but we can only expect changes in capacity to show any consistent correlation with anatomically demonstrable changes.

There are 4 methods of measuring maximum excretory capacity of the liver without catheterization of the hepatic vein. All 4 methods are basically similar; they measure the increment of material retained in the plasma/unit time while it is being injected at a rate well above the capacity of the liver to remove it. These are as follows:

1) *M-H-S Direct Increment Method*. In this method devised by Mason, Hawley and Smith (5) a constant intravenous infusion of BSP is maintained at a rate higher

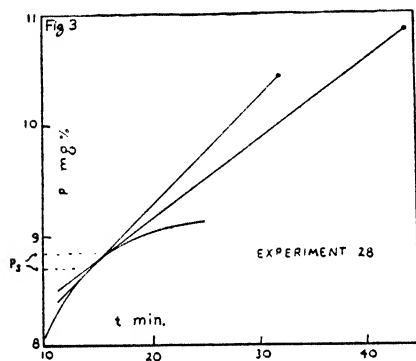


Fig. 3. GRAPHIC METHOD

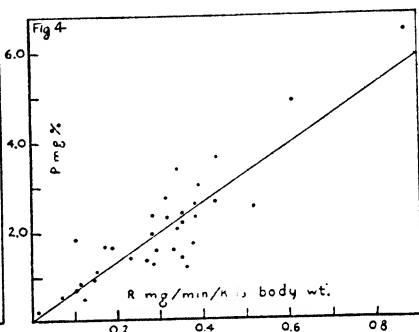


Fig. 4. PLASMA LEVELS AS A FUNCTION OF INFUSION RATES

than the liver's capacity to remove it. Their calculation is summarized in the following equation:

$$Lm = (\text{Infusion rate} - \text{renal excretion rate}) - \frac{V(P - P')}{t},$$

where  $Lm$  = maximum hepatic excretory capacity,  $V$  = plasma volume calculated on the basis of weight,  $P$  and  $P'$  are 2 plasma levels, and  $t$  = the number of minutes elapsed between the 2 plasma levels. We did not employ this method, because of inaccuracy of plasma volumes calculated on a weight basis in rabbits.

2) *Proportional Increment Method*. After determining the values of  $C$  and  $C/V$  on a rabbit, the rate of change,  $r$ , in plasma concentration was determined by taking 3 or more blood samples after 30 or more minutes of constant infusion at a relatively high rate,  $R'$ . The plasma level at which the clearance mechanisms are saturated,  $P_s$ , is calculated from the available data as follows:

$$P_s = \frac{R'}{C} - \frac{C}{Vr}$$

$$CP_s = Lm$$

The relationships of these factors are indicated graphically in figure 1.

3) *Series Increment Method.* In this method, 2 or 3 different relatively concentrated solutions are given in series at a constant rate. The corresponding rates of plasma level increase are a function of the rate of infusion, and  $Lm$  may be obtained by calculation or extrapolation as shown in figure 2.

4) *Graphic Method.* This method is useful when technical errors make it impossible to be objectively certain about the rate of increase in the plasma level after saturation. This situation is depicted in figure 3, where it can be seen that each plasma level after saturation gives an independent determination of  $P_s$  when a tangent to the theoretical time-plasma level curve is drawn from each point.

TABLE I. BSP CLEARANCES OF FED AND FASTED ANIMALS

ANIMAL NO.	CLEARANCE (FED)	CLEARANCE (FASTED)	DIFFERENCE
	cc/min/kg.	cc/min/kg.	
1	13.7	8.5	5.2
2	14.2	9.8	4.4
3	14.2	12.1	2.1
4	21.2	10.1	11.1
5	12.7		
6	16.9	7.3	9.6
7	14.6	12.8	1.8
8	16.7	9.1	7.6
9	16.4	10.1	6.3
10	21.3	9.5	11.8
Mean	16.19	9.92	6.66
Standard Deviation	3.01 (18.6% of mean)	1.71 (17.3% of mean)	1.22

#### MATERIALS AND METHODS

Ten mature male Dutch rabbits were used in this investigation. No anesthesia was required since the animals were restrained in the supine position on an animal board. One ear vein was used for the constant infusion, while samples were withdrawn from the opposite ear vein with syringes moistened with heparin solution to prevent clotting or hemolysis. The time required to withdraw these blood samples varied from 0.5 to 1.5 minutes. The time that blood first appeared in the syringe and the time of withdrawal was recorded to the nearest 0.5 minute and the average of these times taken as the time of the sample. Each blood sample was immediately transferred to a small test tube for mixing with oxalate crystals. The plasma was separated from the cells by centrifuging at 2500 rpm. for 45 minutes in mercury-calibrated Wintrobe tubes, so that the volume of plasma taken for color analysis could be accurately determined. The plasma (0.15–0.30 cc.) was then pipetted into cuvettes to which known volumes of 0.1 N NaOH (1.0–1.33 cc.) had been added. These were then read in a spectrophotometer (Coleman Junior) at 575  $m\mu$  for determinations of BSP content.

The infusion solutions were made from standard ampules of BSP (Hyson, Wescott and Dunning, 50 mg/cc.), diluted with pyrogen-free physiological saline.<sup>2</sup> The rectal temperature of each animal was recorded before and after each experiment; over 35 experiments were performed, and in no instance was there a rise in temperature or noticeable change in thickness of the white cell layer in the Wintrobe tubes.

Constant infusion rates were obtained by driving the plunger of a calibrated 50-cc. syringe with a screw (N.S.  $\frac{1}{8}$  inch–20), turned manually at the rate of one rpm. Determinations of  $C$ ,  $C/V$  or  $Lm$  require from 10 to 15 venepunctures. Scarring and small scab formations over the veins due to shaving and multiple venepunctures made it impossible to use the rabbits at closer than 10- to 14-day intervals.

<sup>2</sup> Supplied through the courtesy of Don Baxter, Inc., Pasadena, California.

## RESULTS

*Exploration of Equilibrium Plasma Levels as a Function of Infusion Rate.* In order to obtain sufficient data on the relationship of clearance to plasma level, the results obtained with 10 rabbits were pooled by plotting  $P$  as a function of  $R$  in terms of mg/minute/kg. body weight. These results are presented in figure 4. Although there is some scattering of points about the mean line, the data justify the conclusion that the line passes through the origin. If the line actually passes to one side of the origin, this deviation is too small to be detected by our methods and too small to invalidate conclusions based on the assumption that the line does pass through the origin. The slope of the line measures clearance, and numerical values for the measure of scatter about this line are given with the data on clearances.

*BSP Clearances of Fed and Fasted Animals.* In table 1, the BSP clearances of the 10 animals are presented. It can be seen that there is considerable variation between determinations on individual rabbits and between separate determinations on the same rabbit. The standard deviation is 3.01 cc/minute/kg. or 18.6 per cent

TABLE 2. FRACTIONAL CLEARANCES OF BSP

C/V, FRACTION OF PLASMA VOLUME CLEARED/MINUTE		
ANIMAL NO.	FED	FASTED
1	.361	.212
2		.227
3		.248
4	.278	.259
6	.295	.179
7		.214
8	.557	.301
9	.238	.207
10	.332	.322
Mean	.344	.219
Standard Deviation	.113 (32.8% of mean)	.052 (23.7% of mean)

of the mean. These determinations were repeated on animals deprived of food for 17 hours. As shown in table 1, the variability is not significantly diminished, but in each instance the measured clearance is definitely lower in the fasting state. The mean decrease is 6.66 cc/minute/kg. The probability of obtaining this consistent decrease by chance is less than 0.01.

*BSP Fluid Compartment.* The volume of the fluid compartment containing BSP was estimated by dividing  $C$  by  $C/V$ . In 9 fasting animals the mean was 41.2 cc/kg., with a standard deviation of 7.38 cc/kg. (18% of the mean). This is of the same order of magnitude as the plasma volumes which can be calculated from reported ranges of blood volume and hematocrit (6). Slightly higher and more variable values were obtained with fed animals, but the difference was not significant.

*Fractional Clearance, (C/V), of BSP.* As indicated in the discussion of theory and equations, early (before 5 minutes) and late (after 5 minutes), falling plasma levels were used to calculate  $C/V$ , and their mean values were compared. Each of these mean values differed from the combined mean by less than one per cent. We may therefore conclude that BSP remains in a single fluid compartment, presumably

plasma. A comparison of fractional clearances obtained from rising plasma levels with those obtained from falling levels reveals a difference of less than 4 per cent from the combined mean. The probability of obtaining this difference by chance is greater than 0.05 and therefore the difference is not significant.

Table 2 presents the fractional clearances of fed and fasted animals together with the statistical properties of the data. The probability of obtaining the observed difference by chance is greater than 0.01, but less than 0.02.

**Maximum Excretory Capacity.** Table 3 presents the measurements of *Lm*. There is no significant difference in the capacity between fed and fasted animals and the standard deviation of the pooled data is 0.104 mg/kg/minute or 10.4 per cent of the mean.

There is evidence that BSP is removed to some small degree by the kidneys, but in our studies with rabbits, we have found that the renal excretion of BSP, even with unusually high blood levels, is so small as to be barely detectable.

TABLE 3. MAXIMUM EXCRETORY CAPACITIES OF BSP

ANIMAL NO.	<i>Lm</i> (FED)	<i>Lm</i> (FASTED)	DIFFERENCE
	mg/min/kg.	mg/min/kg.	
4		.924	
1	.939	.945	.006
6	1.10	1.03	.07
8	1.09	.986	.10
9	1.07	.890	.18
10	1.01	.970	.04
7		1.097	
Mean	1.042	0.977	.076

Combined mean = 1.004 mg/min/kg.

Standard deviation = 0.104 (10.4% mean).

Probability of difference by chance = 0.3.

#### DISCUSSION

Certain facts must be established for each compound used in measuring hepatic clearance. These are: 1) the liver is the sole route of departure from the plasma, or if there are other routes, these are negligible; 2) before removal by the liver the compound remains confined to the plasma, or, if other fluid compartments are entered, equilibrium is rapidly established; 3) clearance is independent of plasma concentration.

The data on fractional clearance indicate that BSP is confined to a single fluid compartment prior to removal from the plasma. If certain cells or spaces other than those involved in excretion are entered, penetration must be either insignificantly small, or if this volume is of significant size there is almost instantaneous equilibration with the plasma. In any event, in the study of normal rabbits the BSP compartment may be considered as representing the plasma volume.

Injection of BSP into eviscerated animals (7) reveals that some BSP may be removed by other tissues (approximately 6%). It is generally believed that the reticulo-endothelial system accounts for this removal (8). It is clear, however, that the liver is certainly the major excretory route, and the available clinical and experi-

mental evidence indicates that variations in rate of BSP removal are correlated with variations in the status of the liver (9). There is no evidence that variations in other tissues produce corresponding variations in BSP removal, but it will be necessary to investigate this possibility before it is possible to interpret pathological BSP clearance data without qualification.

The constancy of  $L_m$  between rabbits and in the same rabbit, whether fed or fasted, is remarkable. The lability of rabbits is notorious, particularly as regards their circulatory responses. The digestive tract in the rabbit is a relatively large one, and no doubt the blood flow to this organ and consequently to the liver is greatly augmented after feeding. The increase in clearance and fractional clearance in fed animals probably is a consequence of this increased blood flow but might be related to the nutritional state of the cell. If the response is caused by increased blood flow, the response might be related either to the filling of additional capillary beds within the liver or to a response of the individual cells. Since  $L_m$  does not change after feeding, the possibility that additional capillary beds are opened and more functional units reached must be ruled out. The constancy of  $L_m$  also rules out the possible role of the nutritional state of the cell in BSP clearance. This does not mean that prolonged fasting and malnutrition might not change this clearance, but refers to the more rapid changes in glycogen storage occurring between feedings. Therefore, the immediate clearance response of the liver is caused by increased blood flow, and this increase is regulated by mechanisms residing in responsive arteriolar beds outside the liver; arterioles within the liver have no appreciable effect in this particular response.

#### SUMMARY

Equations have been derived which analyze the kinetics of clearance and the distribution of compounds injected intravenously. These have been applied to the study of the hepatic clearance of bromsulfalein in fed and fasted normal rabbits. Bromsulfalein is confined to the plasma until excreted by the liver. Below saturation levels hepatic clearance is independent of plasma concentration. Determinations of clearance and maximum excretory capacity indicate that fasting diminishes blood flow to the liver. Hepatic clearance varies with hepatic blood flow, but maximum excretory capacity,  $L_m$ , does not vary with blood flow. Changes in blood flow to the liver in response to feeding or fasting are controlled by arteriolar beds outside the liver and are not appreciably affected by arterioles within the liver.

The author is indebted to Mr. Emmett D. McLaughlin for his assistance in the performance of these experiments.

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# PERIPHERAL CIRCULATORY AND METABOLIC REACTIONS ASSOCIATED WITH ERGOTOXINE HYPER- AND HYPOTHERMIA IN ADULT ALBINO RATS<sup>1</sup>

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**H**YPERTHERMIC reactions of albino rats to intraperitoneal injections of ergotoxine, providing the environmental temperature was 28° C. or higher, and marked hypothermic reactions when the environmental temperature was low (5°-8° C.) have been reported (1). A primary metabolic stimulation, as indicated by increased oxygen consumption, has been shown to be associated with, and apparently in part responsible for, the ergotoxine hyperthermias (2). Evidence has been presented also which supports the generally accepted view that the drug acts directly upon the hypothalamus (3). The respective roles of heat-conservation, heat-production and heat-loss centers of the hypothalamus in the thermal reactions of rats to ergotoxine and the relative importance of central and peripheral actions of the substance have not been conclusively shown.

The present study incorporates an attempt to demonstrate peripheral vascular reactions to ergotoxine alone and in combination with cold stress, as indicated by alterations in skin temperatures. Simultaneous determinations of skin and body temperature, environmental temperature and oxygen consumption have provided information concerning reciprocal relationships among these several factors, all of which are involved in thermoregulation.

## METHODS AND MATERIALS

All rats used in the present investigation were of the Sprague-Dawley strain and all were within the age range of thermoregulation. Body and skin temperatures and the temperatures of the closed chambers within which the animals were confined were continuously recorded during the basal temperature and cold stress phases in each of 31 experiments on 13 control rats. Oxygen consumption was recorded also by means of an electric kymograph and calibrated spirometers. The same group of rats and 6 additional (for a total of 19) were observed in the same manner in 30 separate experiments after the intraperitoneal administration of 4.5 mg/kg. of ergotoxine ethanesulfonate<sup>2</sup> dissolved in 6.25 per cent ethyl alcohol as previously described (2). Ergotoxine-injected rats were placed in the chambers and the recording of temperatures and oxygen consumption started as quickly as possible after injection of the drug.

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<sup>2</sup> Kindly supplied by the Wellcome Research Laboratories, Tuckahoe, N. Y.

Each experiment, whether on a control or ergotoxine-injected rat, consisted of an initial period of approximately 100 minutes during which the chamber temperature was maintained between  $28^{\circ}$  and  $30^{\circ}$  C. by immersion of the chamber in a constant-temperature water bath. At the end of this period the chamber was transferred to a water-containing deep-freeze unit without disturbing the thermopile-animal relationship. The water in the deep-freeze unit was maintained at a temperature of  $11^{\circ} \pm 0.5^{\circ}$  C. by means of a Minneapolis-Honeywell temperature controller. The time in the cold environment varied somewhat but the over-all time of each experiment was at least  $4\frac{1}{2}$  hours.

The use of a 6-channel Brown electronic continuous recording potentiometer, 2 chambers and 2 spirometers facilitated the simultaneous study of 2 animals. Body

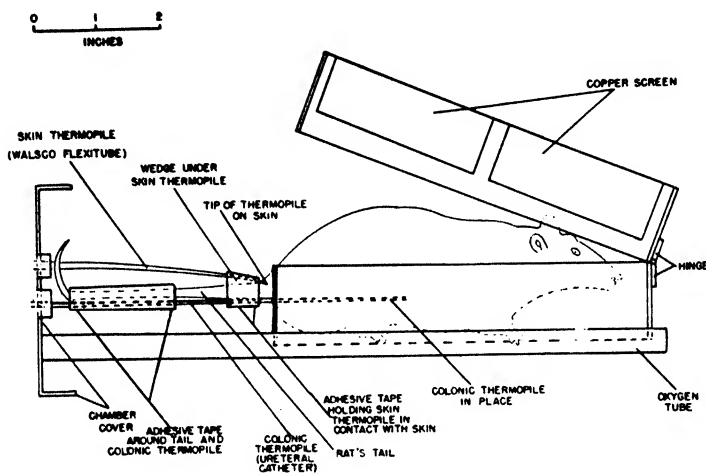


FIG. 1. ANIMAL CONTAINER supported by 2 copper tubes which respectively provide ingress of oxygen to the chamber and egress to a calibrated spirometer. The ureteral catheter is firmly sealed into the cover of the chamber and, after being inserted into the colon, provides a firm anchor for the rat's tail which is taped to it. The skin thermopile is placed in contact with the shaved skin of the rump and also held in place with adhesive tape. In order to assure that the skin thermopile be a measure of the gradient between skin and environmental temperatures, care is taken not to cover its sensitive junctions with tape.

temperatures were measured by means of copper-constantan thermopiles, the sensitive junctions of which were inserted to high colonic levels per rectum and maintained in position by adhesive tape which encircled both the rat's tail and the ureteral catheter through which the copper and constantan wires were threaded (fig. 1). Environmental temperatures were measured by thermopiles which were exposed to the chamber atmospheres (fig. 2). The copper-constantan junctions which comprised the skin thermopiles and which were lightly coated with plastic, were maintained in firm contact with the shaved skin of the rumps of the rats (fig. 2).

Soda lime in porous containers served for the removal of carbon dioxide from the atmospheres of the chambers and condensation of moisture was prevented by the use of similar porous containers filled with anhydrous calcium sulfate (Drierite). It was found also that blotting paper in the bottoms of the containers within which



they were confined effectively prevented the rats from becoming damp from urine; the blotting paper was a necessary precaution even though the containers seemed to be adequately drained.

The relationships of a rat to its container and to the thermopiles and the relationship of the container to the chamber are shown in figures 1 and 2. When a rat had been placed in the container and the thermopiles properly fixed in place, the entire assembly was inserted into the glass chamber which was then screwed to its metal cover.

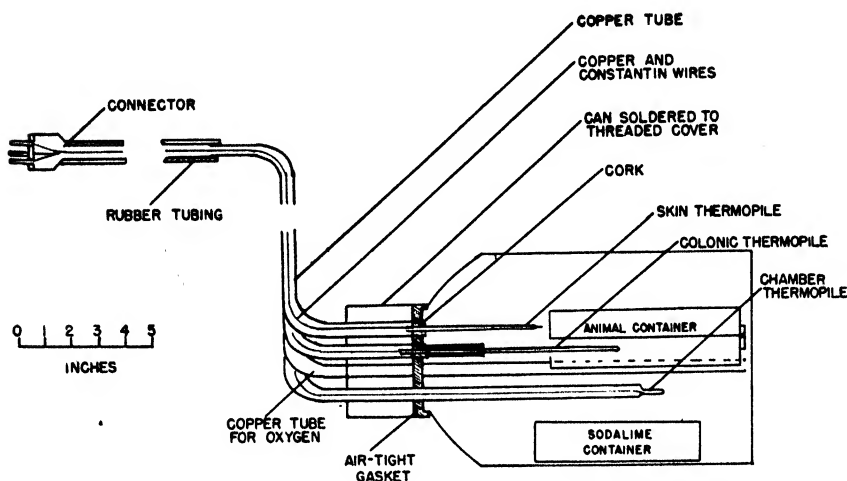


FIG. 2. CHAMBER SCREWED TO ITS METAL COVER WITH CLOSED ANIMAL CONTAINER IN PLACE. THE RELATIONSHIPS OF THE 3 THERMOPILES TO ONE ANOTHER AND TO THE ANIMAL CONTAINER ARE SHOWN. THE LEADS FROM THE THERMOPILES ARE LED OUT OF THE CHAMBER THROUGH COPPER AND RUBBER TUBES TO 6-POST MALE CONNECTORS WHICH ARE PLUGGED INTO CORRESPONDING FEMALE CONNECTORS OF A BROWN ELECTRONIC POTENTIOMETER. ONLY ONE OF 3 SUCH CONNECTORS IS PICTURED. THE RIGHT-ANGLE BENDS IN THE THERMOPILE AND OXYGEN TUBES ALLOW CONVENIENT AND COMPLETE IMMERSION OF THE CHAMBERS IN CONSTANT TEMPERATURE WATER BATHS.

## RESULTS

Hyperthermic reactions to the intraperitoneal injection of ergotoxine varied from moderate to severe; the same was true of the subsequent hypothermias which always occurred when the rats were transferred to the cold environment. A marked hyperthermia and a moderate hypothermic reaction, as they were observed in one single-animal experiment, are illustrated in figure 3; the failure of any significant thermal reaction to appear when the same animal was subjected to identical environmental conditions without ergotoxine administration is evident in this same figure. Average thermal reactions ( $T_i$ ) in all experiments, with and without ergotoxine, are illustrated in figure 4.

An approximation, at least, of the state of the peripheral vessels at any time during a given experiment was arrived at through the use of the 'thermal circulation index' of Burton (4); this index, designated as  $r$ , is equal to  $(T_i - T_o)/(T_i - T_a)$ ,

where  $T_s$ ,  $T_e$  and  $T_b$  are the skin, environmental and body temperatures, respectively. Peripheral vasoconstriction is indicated by lowering of  $r$  values and vasodilatation by their elevation. Rather marked vasoconstriction is therefore readily apparent in the single-animal experiment shown in figure 3 and as an average reaction for the entire group of experiments (fig. 4) during the first 90 minutes after intraperitoneal injection of ergotoxine. Temperatures and  $r$  values, as depicted in figures 3 and 4, are accurately plotted only at 0, 30, 50, 90, 140, 190 and 260 minutes; these points

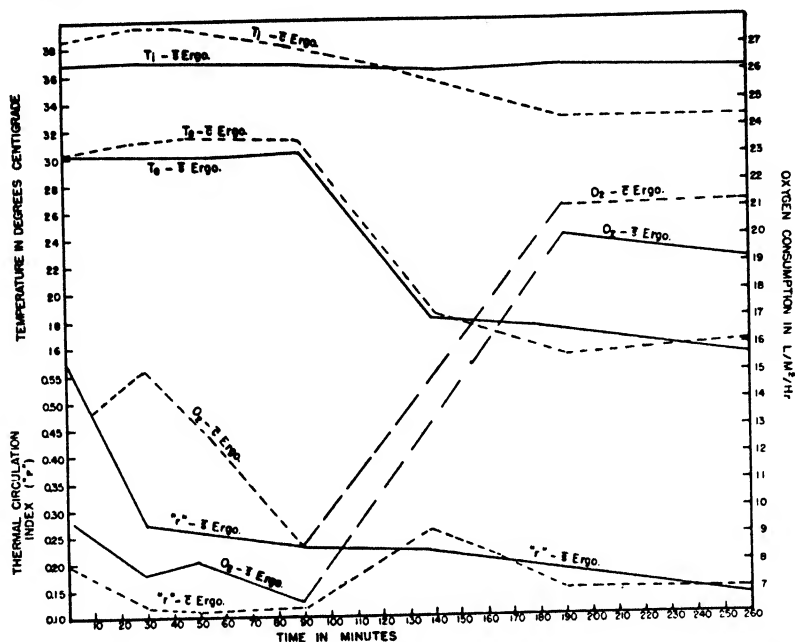


FIG. 3. BODY AND ENVIRONMENTAL TEMPERATURES, thermal circulation indices and oxygen consumption levels as recorded from one animal in 2 experiments—one without and one with ergotoxine. *Solid lines* depict the reactions without ergotoxine and the *broken lines* those following intraperitoneal injection of 4.5 mg/kg. of ergotoxine ethanesulfonate. It may be noted that the hyperthermic reaction to ergotoxine was already well established when recording began. Values are accurately plotted at 0, 30, 50, 90, 140, 190 and 260 minutes; these points are connected by straight lines which serve to indicate upward or downward trends during the intervening periods.  $\bar{e}$  and  $\bar{s}$  Ergo. = with and without Ergotoxine, respectively;  $T_b$  = body temperature;  $T_e$  = environmental temperature;  $O_2$  = oxygen consumption;  $r$  = thermal circulation index.

are connected by straight lines which merely serve to indicate the relative rise or fall which occurred during the intervening period.

Peripheral vasodilatation resulted when rats were given ergotoxine, allowed to undergo hyperthermic reactions and then transferred quickly to the cold environment (figs. 3 and 4). This reaction of the peripheral vascular system to cold stress, after ergotoxine injection, was in marked contrast to the vasoconstriction characteristic of uninjected animals (fig. 4).

Average oxygen consumption levels at basal temperatures tended to be quite

constant at approximately  $10 \text{ l/m.}^2/\text{hour}$  in the group of control experiments (fig. 4). The average level of oxygen consumption 30 minutes after injection of ergotoxine was  $12.2 \text{ l/m.}^2/\text{hour}$  (fig. 4). The falls in body temperature which occurred during the interval between 30 and 90 minutes after ergotoxine was given were associated with significant decreases in oxygen consumption levels.

Exposure to the cold environment stimulated marked increases in oxygen consumption levels in all non-ergotoxine experiments (figs. 3 and 4). The average oxygen consumption in the cold environment, for all experiments in which ergotoxine was

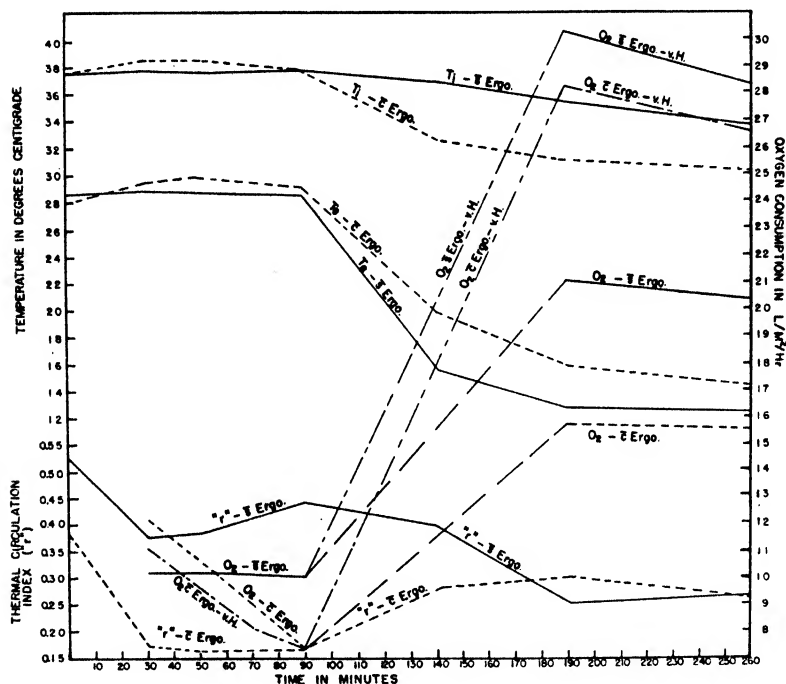


FIG. 4. AVERAGE BODY AND ENVIRONMENTAL TEMPERATURES, thermal circulation indices and oxygen consumption levels, with and without ergotoxine, for all the experiments carried out. Oxygen values adjusted through application of van't Hoff's law are shown also ( $\text{O}_2 \bar{c}$  Ergo., v. H. and  $\text{O}_2 \bar{s}$  Ergo., v.H.). Abbreviations are the same as in figure 3.

administered, was considerably less than in non-ergotoxine experiments ( $15.5 \text{ l/m.}^2/\text{hour}$  as against  $20.3$  at 260 minutes). Oxygen consumption rates were not calculated for the period between 90 and 190 minutes when accurate corrections for the rapidly changing environmental temperatures were impossible. The broken lines extending between these points in figures 3 and 4 do not, therefore, represent actual levels of oxygen utilization by the animals. It also was impossible to obtain accurate oxygen consumption values during the first 30 minutes of the experiments in most cases. In those cases where this was accomplished a sharp rise occurred in ergotoxine-injected rats during the period of the hyperthermic response (fig. 3).

## DISCUSSION

The hyperthermic responses of rats to ergotoxine, on the basis of the thermal circulation indices and oxygen consumption levels presented in figures 3 and 4, appear to result from a combination of peripheral vasoconstriction and metabolic stimulation which are respectively responsible for diminished heat loss and increased heat production. The increase in metabolism, indicated by increased oxygen consumption, can be attributed to direct stimulation of hypothalamic centers (3). The initial vasoconstriction (during the period of rising body temperature) may be due to hypothalamic stimulation but the sustained vasoconstriction, evident between 30 and 90 minutes after administration of ergotoxine, when the rapid decline in oxygen consumption (figs. 3 and 4) suggests a substantial decrease in hypothalamic stimulation, is probably due mainly to direct action of the substance on the smooth musculature of the peripheral vessels (5).

It is quite apparent that initial vasoconstriction also occurs without ergotoxine in rats subjected to insertion of colonic thermopiles and confinement in the chambers (figs. 3 and 4). It is logical to assume that this is due to secretion of epinephrine (6) with direct stimulation of peripheral vessels by this substance. The quantity of epinephrine liberated into the blood stream as a result of thermopile stress apparently is not sufficient in ergotoxine-injected rats for the initiation of the epinephrine reversal phenomenon described by Dale (5) and others since, as noted above, peripheral vasoconstriction persists in these animals (fig. 4).

Transfer of ergotoxine-injected rats to the cold environment appears to be responsible for prompt sympathetic stimulation and increased hormonal epinephrine, of sufficient amount to induce epinephrine reversal and marked vasodilatation. When control animals are exposed to the same environment they respond with a typical heat-conserving vasoconstriction of peripheral vessels. The marked contrast between the peripheral vascular reactions to cold stress of control rats and of those receiving ergotoxine is readily apparent in figure 4 ( $r \bar{s}$  Ergo. and  $r \bar{c}$  Ergo.). The most rapid reductions in body temperatures of rats which were ergotoxine-injected and cold-stressed (fig. 4,  $T_i \bar{c}$  Ergo.) occurred during the period of rapidly increasing vasodilatation (beginning of cold stress to 190 minutes) and this suggests that there is inability of heat-production mechanisms to compensate for the profound heat loss occasioned by peripheral vascular atony.

Failure of ergotoxine-injected animals to attain the relatively higher level of oxygen consumption characteristic of control rats (fig. 4) is not as paradoxical as it may appear, if it is considered in the light of van't Hoff's law.<sup>3</sup> The rapid and pronounced vasodilatation associated with epinephrine reversal apparently accounts for a sufficiently severe fall in body temperature to induce a kinetic reduction in reaction rates of enzyme systems essential to heat production. When the temperatures of control and ergotoxine-injected rats at 190 and 260 minutes from the beginnings of the experimental records were taken into consideration and the oxygen consumption

<sup>3</sup> The van't Hoff or Arrhenius Law is an empirical relation between the reaction rate and the temperature of the reaction environment (the log of the reaction rate is related to the reciprocal of absolute temperature). It is used here as an aid to interpretation and represents only an approximation of the effect of reduced body temperature on the animals' total metabolism.

values adjusted upward through utilization of the van't Hoff equation, as previously described (2, 7), to the levels which should have existed if body temperatures had been maintained within the normal range, the adjusted values (fig. 4,  $O_2 \bar{s}$  Ergo., v. H. and  $O_2 \bar{c}$  Ergo., v. H.) for the 2 groups of experiments were of essentially the same magnitude (6.6% difference between controls and experimentals at 190 minutes and 6.3% difference at 260 minutes). It appears likely therefore that ergotoxine-injected rats are capable of approximately as great a metabolic effort as control animals but that they are kept from it by peripheral vasodilatation and consequent failure of body temperature to be maintained.

That the average fall in body temperatures of control rats (fig. 4) was greater than in the single animal represented by figure 3 and that oxygen consumption was correspondingly lowered, through operation of van't Hoff's law, is probably explained by the fact that blotting paper was not used in the animal containers in some of the earlier experiments; in a few experiments, therefore, the rats became damp and did not maintain their body temperatures as well as is customary for dry animals.

Application of the van't Hoff equation to oxygen consumption levels during the period of hyperthermia (30 minutes after beginning of experimental records) indicates that the recorded increase in consumption at this time reflects a greater metabolic response than would have been occasioned by the increase in body temperature. The actual increase in oxygen consumption of ergotoxine-injected rats over controls was double that which would have been anticipated through operation of van't Hoff's law or 16.4 per cent as compared to 8.1 per cent. It must be noted, in connection with the consideration of 30-minute oxygen consumption levels, that the van't Hoff relation is much more reliable within the body temperature range recorded at 30 minutes than within the lower and wider ranges at 190 and 260 minutes; therefore the 100 per cent increase in actual oxygen consumption level over that expected from operation of van't Hoff's law certainly indicates that primary metabolic stimulation is one of the factors concerned in ergotoxine hyperthermia. The increase in metabolism and the accompanying vasoconstriction result in hyperthermia as the manifestation of activity of both heat-production and heat-conservation mechanisms; both may be activated by direct stimulation of appropriate centers in the hypothalamus, but direct action of ergotoxine on peripheral vessels must be included among the factors responsible.

#### SUMMARY

Simultaneous recording of body and skin temperatures and oxygen consumptions of Sprague-Dawley albino rats, with and without ergotoxine and under basal and cold-stress environmental conditions, has led to the conclusion that ergotoxine hyperthermia is due to a combination of peripheral vasoconstriction and increased metabolic activity. Peripheral vascular reactions were evaluated through the use of the 'thermal circulation index' of Burton. Both the heat-conservation and heat-production mechanisms are believed to be activated through direct stimulation of hypothalamic centers by ergotoxine. Vasoconstriction, continued beyond the height of the hyperthermic reaction and associated with a rapid decrease in metabolism, as indicated by reduced oxygen consumption, is thought to be due to direct action of the

drug upon the smooth musculature of peripheral vessels. Uninjected rats responded to cold stress by prompt and sustained peripheral vasoconstriction while those given ergotoxine intraperitoneally exhibited marked vasodilatation which was interpreted as being due to epinephrine reversal. The peripheral vasodilatation of ergotoxine-injected rats, under cold stress, resulted in a marked reduction in body temperature; this, in turn, appeared to effect a kinetic reduction in reaction rates of enzyme systems essential to heat production with consequent failure of such animals to use oxygen at as high a level as control animals subjected to the same environment and capable, through active vasoconstriction and increased metabolism, of maintaining their body temperatures at normal or near normal levels.

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# ACTION OF INSULIN ON THE 'PERMEABILITY' OF CELLS TO FREE HEXOSES, AS STUDIED BY ITS EFFECT ON THE DISTRIBUTION OF GALACTOSE

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ON THE basis of work from this laboratory, we had previously concluded that, in so far as the extrahepatic tissues were concerned, insulin promoted the rate of entry of glucose especially at lower blood glucose levels. In the absence of insulin, one could force the entry of more glucose into the tissues by raising its level in the blood (1-3). In common with other investigators, we thought that this action of insulin was exerted upon one of the enzymatic steps by which glucose is brought into the metabolic cycle of the cell (4). The most likely site of action would be one exerted directly or indirectly upon the rate of phosphorylation of the sugar to glucose-6-phosphate. Recently, Cori and co-workers found that insulin increased the *in vitro* activity of muscle hexokinase, previously inhibited by certain pituitary and adrenal cortical substances (5-7). This type of action is at variance with the well known hypersensitivity of the hypophysectomized and adrenalectomized animal to the action of insulin *in vivo*. It has also been found difficult to demonstrate this action at will or to show its specificity (8-10). Tissues from hypophysectomized animals are at least as sensitive to the action of insulin as are tissues from normal animals, despite the absence of the postulated hexokinase inhibitor (11). Positive actions of insulin on oxygen consumption, pyruvate metabolism etc. have been obtained (12-14) and denied (3, 13, 15, 16).

It has not been proven that the rate of withdrawal of glucose from blood depends primarily upon the activity of the glucose-phosphorylating system. This would assume that the penetration of glucose into the cell is not a limiting factor or that the primary phosphorylation is itself the means whereby glucose is brought into the cell. It is quite possible, however, that an active glucose transfer mechanism exists which serves to bring the free sugar into the cell where it is subsequently subjected to the action of glucokinase. Insulin may act by facilitating such a transfer mechanism. It is obviously not feasible to study this type of effect by using glucose as the substrate, since the over-all rate of glucose disappearance is the result of the rate of penetration and the rate of transformation. We, therefore, selected a close

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chemical relative of glucose which under certain conditions would not be metabolized. Such a substance is galactose which is transformed to glucose in the liver, kidneys and probably the intestinal tract (17-19). Skeletal muscle or muscle extracts *in vitro* do not seem to possess any galactokinase activity (17, 20). Accordingly, we studied the effect of insulin on the rate of distribution of a given amount of galactose in animals previously eviscerated and nephrectomized. In this manner, the rate at which this sugar enters tissues could be studied independently of its rate of metabolism (21). It will be shown that insulin exerts a marked effect upon the rate with which galactose leaves the blood and enters tissues despite the fact that no transformation of galactose occurs in the tissue itself.

#### METHODS

Dogs were eviscerated and nephrectomized under sodium pentobarbital anesthesia. Maintenance of blood pressure, heart rate, easy slow respirations and color of the mucous membranes were used as criteria of an adequately functioning preparation during the 3 to 5 hours of observation. Blood glucose levels were maintained by the constant infusion of  $\frac{1}{4}$  gm. of glucose/kg/hour in saline (about 26 cc/hour). In certain of the experiments insulin was added to this infusion at the rate of 1, 2 or 4 units/kg/hour after an initial priming dose of 5 units i.v. The test substances (glucose, galactose, sucrose, urea, creatine) were injected i.v. in a volume of 50 cc. of saline, over a 2- to 3-minute period. Arterial blood samples were taken at intervals during the 3 to 5 hours of the experiment for assay of the blood levels of these test substances.

Rats were eviscerated and nephrectomized by a similar one-stage technique. Some of the rats were anesthetized with sodium pentobarbital and others with ether. The etherized animals quickly recovered and moved about the cage, requiring further anesthesia when killed. No difference was observed between the groups despite the use of different anesthetic agents. Galactose was injected into the jugular vein in a single test dose, while glucose with or without insulin was injected s.c. at hourly intervals during the experiment. At the end of one-hour and 2-hour periods the animals were killed by withdrawing blood from the abdominal aorta for blood level determinations. The carcass was then quickly severed into small pieces, frozen in a dry ice-acetone mixture and ground into a powder with dry ice. The powdered carcass was then extracted with water and examined for its content of galactose.

Glucose was determined by the method of Shaffer and Somogyi (22); galactose by the method of Geyer (23); reducing power was determined before and after fermentation with baker's yeast in order to remove the glucose; sucrose by the method of Morris (24); urea by the method of Ormsby (25) and creatinine by the method of Folin and Wu (26).

#### RESULTS

The eviscerated-nephrectomized dog when given a single injection of glucose exhibited a continuous and progressive fall in blood level as the tissues continued to take up and utilize the glucose. By contrast, urea in this preparation was neither produced, excreted or utilized in the absence of liver and kidneys. An injection of



urea was quickly distributed through the tissues and the blood level fell to a value corresponding to distribution in total body water (about 70%) in 30 to 60 minutes. The blood urea then maintained an unchanging level. Galactose under these conditions behaved like urea, i.e. as a non-utilized, non-excreted substance distributing itself through the tissues and then maintaining a constant level. Figure 1 represents 3 such experiments using one gm/kg. of glucose, galactose and urea, respectively, in 3 different preparations. The plasma level of glucose fell from a value of 690 mg. per cent, 5 minutes after injection to a level of 160 mg. per cent in 4 hours. Urea was distributed very quickly and the 5-minute level of 200 mg. per cent fell to 152 mg. per cent by 30 minutes and was maintained at this level for the 4 hours of observation. Galactose like glucose exhibited a high initial value of 529 mg. per cent, falling to 311 mg. per cent by 90 minutes and maintaining a steady level of about 290 to 295 mg. per cent for the next 2½ hours. The non-utilization of galactose was further tested by means of recovery experiments in rats, as described below.

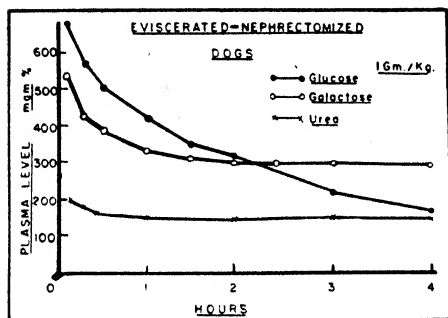


FIG. 1. THE SUBSTANCES TESTED were given in a single injection at 0 time. Note the continuous fall of the plasma glucose level due to its utilization. By contrast the injected urea is quickly distributed in body water, and its level remains stationary, since it is not being produced or used. Galactose behaves like urea; it is not being utilized in the absence of the liver, kidneys and gastrointestinal tract.

The effect of insulin upon this distribution of galactose was then tested. In a series of 8 dogs, single injections of one gm/kg. of galactose were administered after evisceration-nephrectomy. To 4 of these preparations a priming dose of 5 units of crystalline zinc insulin was given and insulin added to the constant infusion of glucose at the rate of 1, 2 or 4 units/kg./hour. In these 4 experiments the galactose was administered 15 minutes after the beginning of insulin infusion. Figure 2 represents the resulting blood curves for galactose. In the absence of insulin a blood level of about 230 mg. per cent was exhibited after 1½ hours and maintained thereafter. This represents a 'galactose space' of about 45 per cent of body weight. The addition of insulin in any of the above dosages resulted in a sharp divergence of the curve from the onset and the establishment of a much lower maintenance level of about 130 mg. per cent. The variations of individual experiments from each other are small. The insulin and non-insulin curves are widely separated. One of the non-insulin group was a severely diabetic animal (pancreatectomy). As far as the distribution of a single injection of galactose is concerned there is no apparent difference between an insulin-free preparation and one containing the normal amount of endogenous insulin. This similarity of depancreatized preparations to those possessing

only endogenous insulin was seen in 2 other experiments.<sup>3</sup> There was also no difference exhibited between 1, 2 or 4 units of insulin/kg./hour. At all these dosages the final level of galactose observed corresponded to a distribution in approximately 70 per cent of body weight and is of the same order as the level reached by a comparable amount of urea. Since this corresponds to the figure generally accepted for total body water, the maximal effect of insulin on distribution of galactose throughout the tissues was exhibited, and there can be no greater influence of 4 units over one unit.

These same phenomena were demonstrated at smaller dosages of  $\frac{1}{2}$  gm. and  $\frac{1}{4}$  gm. of galactose/kg. with the establishment of essentially steady levels after 90 minutes and with marked divergence of the curves with addition of insulin. Using  $\frac{1}{4}$  gm/kg. a final level of 60 mg. per cent was obtained without insulin, a value just  $\frac{1}{4}$  of that obtained with the corresponding dose of one gm/kg., as one would expect in dealing with the distribution of a non-utilized substance.

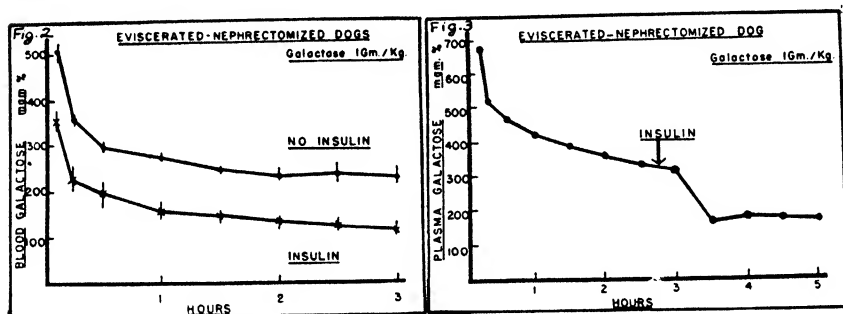


FIG. 2. GALACTOSE WAS INJECTED AT 0 TIME. Note that insulin caused a faster and wider distribution of the galactose. The galactose 'space' in the absence of added insulin was about 45 per cent of body weight; in the presence of added insulin this space widened to about 70 per cent of body weight. The vertical lines indicate range of variation of all values obtained.

FIG. 3. GALACTOSE WAS INJECTED AT 0 TIME. The injection of insulin was begun 2 hours later and continued for the remainder of the experiment. Note that the blood galactose level fell sharply, reached an approximately 70 per cent body weight distribution and was then maintained.

In 3 experiments insulin was withheld for  $1\frac{1}{2}$  or  $2\frac{1}{2}$  hours and then given in the same manner as described above (a priming dose plus constant infusion). In all 3 instances a sharp fall in level was exhibited. This obtained with one gm/kg. and  $\frac{1}{2}$  gm/kg. of galactose and hence at both high and low levels. One of these experiments is illustrated in figure 3.

The galactose determinations were done using whole blood. Determinations on plasma gave comparable results although at higher levels since the red blood cells of the dog are essentially impermeable to galactose. An example of one of the experiments done on plasma is shown in figure 1. The results with plasma were, however, much more irregular with greater variation between experiments. This was found to

<sup>3</sup> By a somewhat different procedure which involves much lower blood galactose levels, we can demonstrate the effect of smaller doses of insulin. Under these conditions there is a significant difference between the behavior of depancreatized animals and animals possessing only endogenous insulin.

be due to an increasing fragility of red blood cells developing with time in the eviscerate preparation. In the process of separating cells and plasma, galactose would enter the red cells in a capricious fashion. Thus, although the content per unit volume of blood was unchanged, in the process of obtaining the sample, centrifuging and separating plasma, a variable amount of galactose might enter the red cells yielding falsely irregular curves.

Simultaneous blood glucose levels in all these experiments failed to reveal any consistent relation of glucose changes to the effect observed on galactose. To further demonstrate that the 'galactose effect' was not due to some secondary phenomenon arising from any action of insulin upon blood glucose, infusion of glucose was omitted after evisceration in 2 dogs. Artificial respiration was instituted and both vagi were anesthetized with procaine and severed in the neck. In this fashion, the fatal effects of hypoglycemia were obviated and after  $1\frac{1}{2}$  hours when the blood glucose level was 2 mg. per cent, insulin was infused and a priming dose given as in previous experiments. Galactose, one gm/kg., was then injected. The resultant curve of blood galactose levels was similar to previous experience, despite the negligible blood glucose

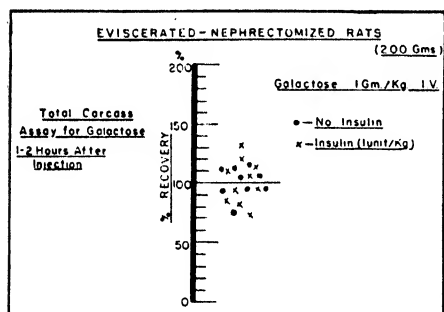


FIG. 4. NOTE THAT THE FIGURES FOR RECOVERY of the injected galactose are randomly distributed around the 100 per cent level, for both the insulin and non-insulin group. Average recovery: non-insulin group 103 per cent  $\pm$  13 (S.D.); insulin group 100 per cent  $\pm$  19 (S.D.).

levels. Thus the effects observed in our experiments were the results of a direct insulin action on galactose and not secondary to the hypoglycemic effect of the hormone.

Although galactose appeared to be behaving as a non-utilized substance, recovery experiments were attempted to prove the point. Since whole carcass analysis for galactose is impractical in the dog, rats were used for this purpose. Conditions of the experiments and dosage of galactose etc. were similar to those used for the dog in terms of body weight. Eviscerated-nephrectomized rats receiving insulin exhibited blood galactose levels which were about 100 mg. per cent lower than in the animals without added insulin. These results were obtained after both one- and 2-hour periods. The effect of insulin on galactose distribution in the rat was thus similar to that seen previously in the dog. In contrast to the differences in blood levels, the total content of galactose in the carcass was the same whether or not insulin had been administered. The 9 animals without insulin and the 10 animals receiving insulin yielded recoveries of galactose averaging 100 per cent. There is, however, considerable variation ( $103\% \pm 13^4$  for the non-insulin group, and  $100\% \pm 19^4$  for the insulin

<sup>4</sup> Standard deviation.

group) arising from the inherent difficulties of tissue analysis for specific carbohydrates. As can be seen in figure 4, there is no difference in the recoveries of galactose from the insulin group as compared to the non-insulin group despite the marked divergence in blood levels.

The effect of insulin on the distribution of several other substances was tested in the eviscerated-nephrectomized dog. It was found that the distribution of urea, creatinine and sucrose was unaffected by the administration of insulin.

#### DISCUSSION

We have in these studies dissociated utilization or transformation of a hexose from its distribution and shown that there is a marked effect of insulin in promoting the movement of unchanged galactose into cells. In the various schemes and theories of insulin action on the enzyme apparatus of glucose metabolism, movement of glucose into the cell is assumed to be a relatively unhindered phenomenon, playing no limiting role in controlling the rate of cellular utilization. It would seem highly unlikely that insulin would be acting on an intracellular enzyme with respect to glucose while acting to promote the transfer in an unaltered form of so closely related a substance as galactose. At least for galactose, insulin is necessary for free access of the hexose to the intracellular space.

Although tissue analysis for specific carbohydrates has serious limitations, our recovery experiments failed to show any difference between animals receiving insulin and those without it. The differences in blood levels attained could not be attributed to a disappearance of galactose from the preparation with the addition of insulin. Previous studies (17-20) have failed to reveal enzyme systems capable of transforming galactose except in those organs excluded from this preparation (liver, kidneys and intestines). And finally, the establishment of an essentially constant blood level is what one would expect of a substance which is neither produced, utilized or excreted.

If galactose is then unchanged in the eviscerated-nephrectomized animal, once distribution is complete and a constant level is established, a decrease in blood level must reflect movement of the substance into the intracellular space. Insulin at any point promoted further movement into cells and the establishment of lower blood levels. At the dosage employed in these studies (1, 2, and 4 units/kg.) this movement is maximal and the blood levels indicate a distribution through 70 per cent of body weight; i.e. through total body water. The fact that no further depression of blood level is obtained with more insulin serves as further evidence that distribution and not utilization is being affected here. The implications of these observations allow us to set up a hypothesis of insulin action. The entry of glucose into the metabolic scheme has been proposed as the crucial point where insulin might act, explaining the variety of end-results described on glycogen, fat, nitrogen balance, ketosis etc. To date, attention has centered on the enzymatic phosphorylations, which bring glucose into the metabolic scheme. In the case of galactose, there are no enzymes in the tissues of our preparation which can act upon the galactose. Insulin nevertheless promotes its transfer into the cell until the intracellular concentration approaches that outside the cell and galactose is equally distributed in body water. We are proposing

that transfer of glucose into the cell is similarly not a free and unlimited process but rather an important limiting factor upon which insulin acts to promote entry. The subsequent fate of the glucose will depend on the presence and state of activity of the enzyme apparatus.

#### SUMMARY

Dogs and rats were eviscerated and nephrectomized and given intravenous injections of glucose, galactose, urea, creatinine and sucrose. Blood levels for glucose revealed a continuous fall in keeping with the continuous utilization of glucose. The other substances established constant blood levels within  $1\frac{1}{2}$  hours in keeping with the distribution of non-utilized, non-excreted substances. Insulin resulted in the establishment of lower galactose blood levels which were again essentially constant. Urea, creatinine and sucrose levels were unaffected by insulin. Recovery experiments have failed to reveal any evidence for utilization of galactose in the absence of liver, kidney and intestine. These organs were excluded in our experiments. Insulin acts to promote the distribution of galactose and not its utilization. Maximal effect of insulin occurred when distribution had reached 70 per cent of body weight, i.e. total body water. A hypothesis of insulin action is proposed which attributes to insulin the role of facilitating the rate of transfer of some hexoses into the cell, as opposed to concepts of a direct effect on the rates of action of intracellular enzymes.

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## VITAMIN B<sub>12</sub> CONTENT OF BLOOD FROM VARIOUS SPECIES<sup>1,2,3</sup>

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**S**IMULTANEOUSLY with the isolation of vitamin B<sub>12</sub> (1, 2) West (3) announced that the administration of the vitamin to pernicious anemia patients produced an increase in reticulocytes, red cell count and hemoglobin. Numerous reports have appeared to confirm the latter observation (4). The vitamin content of blood from various species has been determined and has been shown to be related to the content of such vitamins in the diet. Schweigert and Pearson (5) determined the folic acid content of blood from various species and Schweigert *et al.* (6) and Schweigert (7) reported that the level of folic acid in the blood of turkeys varied with the dietary treatment. Greenberg and Rinehart (4) stated that the thiamin content of human blood was relatively static and was not appreciably affected by changes in thiamin intake. Sheahan (8) determined the ascorbic acid content of blood serum from cows, bulls, sheep and pigs and noted that the level of ascorbic acid in the serum varied with the season. Lindley *et al.* (9) found that there was a decrease in the vitamin A and in the ascorbic acid content of blood from vitamin A deficient rams. With the above facts in mind it appeared desirable to determine the vitamin B<sub>12</sub> content of the blood from various species.

### EXPERIMENTAL

Samples of blood were collected in oxalated tubes from rabbits, rats, mice, cattle, sheep, goats, swine, turtles, chickens, turkeys, humans, dogs and horses. The samples were shaken thoroughly and an aliquot of whole blood was taken for the determination of vitamin B<sub>12</sub>. Six ml. of whole blood was pipetted directly into 50 ml. of H<sub>2</sub>O in a 125-ml. Erlenmeyer flask. Ten ml. of a pancreatin solution (3 mg/cc.) were added and the solution was covered with toluene. The flask was stoppered with cork and placed in a shaker at 37.5°C. for approximately 24 hours at

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<sup>2</sup> The crystalline vitamin B<sub>12</sub> used in the microbiological assays was generously supplied by Dr. D. F. Green, Veterinary Department, Merck & Co., Inc., Rahway, New Jersey.

<sup>3</sup> A preliminary report of this work was given at the 117th meeting of the American Chemical Society in Houston, Texas, March 29, 1950. Attention is directed to the fact that the level of B<sub>12</sub> in the blood shown in the abstracts of papers (117th meeting A.C.S.) should be in  $\mu\mu\text{g.}$  and not  $\mu\text{g.}$  per ml. of blood.

the end of which time the samples were autoclaved for 5 minutes at 15 pounds pressure. After cooling, the samples were made up to 100 ml. and filtered. In some instances one-half of this filtrate (50 ml.) was adjusted to pH 12.0, autoclaved for one hour and the pH was adjusted to 6.8-7.0. These stock solutions were covered with toluene and stored at approximately 20°C. Dilutions for the microbiological assay for vitamin B<sub>12</sub> were made from the above stock solutions. The vitamin B<sub>12</sub> activity of the samples was determined according to the procedure of Skeggs *et al.* (10) with the thiomalic acid as the reducing agent in the medium. *Lactobacillus leichmannii* 4797 (ATCC) was the test organism and acid production was the criterion used to measure growth response. Crystalline vitamin B<sub>12</sub> was used as the standard in all of the assays mentioned in this report.

#### RESULTS AND DISCUSSION

It is readily apparent that the vitamin B<sub>12</sub> content of whole blood varies with the species. The vitamin B<sub>12</sub> content of whole blood from the human, dog, white rag, cow, sheep, goat and pig varied from 0.5 to 1.0  $\mu\text{g}/\text{ml}$ . No explanation can be given for the fact that cotton rat blood contained five times as much vitamin B<sub>12</sub> as did that of the white rat when both were maintained on approximately the same diet. The Swiss albino mouse blood is only slightly higher in vitamin B<sub>12</sub> than that of the various animals listed above. No explanation is offered as to why the blood of the American albino mouse contained more vitamin B<sub>12</sub> than did that of the Swiss albino mouse since both were fed the same diet. It should be pointed out that a composite sample of blood from 20 mice made up each sample shown in table 1. It is interesting to note that the blood from cattle on pasture during a dry season (table 1) contained less vitamin B<sub>12</sub> than did that of calves on a fattening ration in a dry lot. However, attention is again directed to the rather constant vitamin B<sub>12</sub> level in the blood of the human, dog, white rat, calf (dry lot fed), sheep, goat and pig. The vitamin B<sub>12</sub> content of horse blood is over two times as high as that of the human, sheep, dog, pig and white rat. No explanation is offered for the fact that horse blood contained more B<sub>12</sub> than some of the species mentioned above. The B<sub>12</sub> content of rabbit blood is particularly high when compared to that of the human, dog, sheep, goat, pig and calf (table 1) and is probably traceable to the nocturnal coprophagy which the rabbit is known to practice (11). The vitamin B<sub>12</sub> content of turtle, chicken and turkey blood is higher than that of other species with the exception of the rabbit (table 1). The blood of the turtle, chicken and turkey has nucleated erythrocytes (12) and it is possible that a part of the apparent vitamin B<sub>12</sub> activity in the blood of these animals might be due to thymine desoxyriboside since *L. leichmannii* responds to thymidine (10, 12, 13). It has been shown that vitamin B<sub>12</sub> is destroyed by autoclaving in an alkaline medium (13, 14). Accordingly, samples of blood from the cotton rat, white rat, American albino mouse, Swiss albino mouse, calf, sheep, goat, rabbit, turtle, chicken and turkey were autoclaved at pH 12.0 for one hour. The apparent vitamin B<sub>12</sub> activity, as measured by *L. leichmannii* 4797, after autoclaving is shown in table 2. The vitamin B<sub>12</sub> content of blood from all species tested was destroyed with the exception of that from the turtle, chicken and turkey. It is believed that the remaining *L. leichmannii* activity of the blood from the turtle, chicken and turkey after autoclaving was due to the presence of

thymidine which would probably exist in greater concentration in the blood of these animals due to the fact that the erythrocytes are nucleated. It is also interesting to note that the vitamin B<sub>12</sub> activity which was destroyed by autoclaving turtle, chicken and turkey blood in an alkaline medium was within the range of the vitamin B<sub>12</sub> content of the blood of other species reported in table 1, with the exception

TABLE 1. VITAMIN B<sub>12</sub> CONTENT OF WHOLE BLOOD FROM VARIOUS SPECIES AS MEASURED BY *Lactobacillus leichmannii*

SOURCE OF SAMPLES	NO. OF SAMPLES	VITAMIN B <sub>12</sub> CONTENT		SOURCE OF SAMPLES	NO. OF SAMPLES	VITAMIN B <sub>12</sub> CONTENT	
		Range	Average			Range	Average
Human.....	10	0.6-1.4	0.8	Sheep.....	4	0.6-0.9	0.7
Dog.....	4	0.5-1.1	0.9	Goat.....	4	0.5-0.9	0.7
Cotton rat.....	3	3.3-3.8	3.6	Pig.....	10	0.8-1.3	1.0
White rat.....	26	0.5-1.2	0.8	Horse.....	6	1.2-3.4	2.1
American albino mice.....	2	2.2-2.3	2.3	Rabbit.....	13	6.4-15.0	10.1
Swiss albino mice.....	2	0.9-1.4	1.2	Turtle.....	5	5.9-6.9	6.6
Calf (dry lot fed).....	6	0.8-1.0	0.9	Chick.....	15	3.8-6.6	5.6
Cow (pasture fed).....	8	0.4-0.5	0.5	Turkey.....	10	4.5-6.0	5.3

Values expressed as  $\mu\text{g.}$  vitamin B<sub>12</sub> activity/ml.

TABLE 2. EFFECT OF AUTOCLAVING ONE HOUR AT pH 12.0 ON THE APPARENT VITAMIN B<sub>12</sub> CONTENT OF WHOLE BLOOD FROM VARIOUS SPECIES AS DETERMINED WITH *Lactobacillus leichmannii* 4797

SOURCE OF SAMPLES	NO. OF SAMPLES	VITAMIN B <sub>12</sub> CONTENT		ACTIVITY DESTROYED BY AUTOCLAVING
		Before Autoclaving	After Autoclaving	
Cotton rat.....	3	3.6	0.0	3.6
White rat.....	3	0.7	0.0	0.7
American albino mice.....	2	2.3	0.0	2.3
Swiss albino mice.....	2	1.2	0.0	1.2
Calf.....	6	0.9	0.0	0.9
Sheep.....	4	0.7	0.0	0.7
Goat.....	4	0.7	0.0	0.7
Rabbit.....	13	10.1	0.0	10.1
Turtle.....	5	6.6	4.6	2.0
Chick.....	5	5.8	4.8	1.0
Turkey.....	5	5.3	3.8	1.5

Values expressed as  $\mu\text{g.}$  vitamin B<sub>12</sub> activity/ml.

of the rabbit. From these assumptions it might be stated that the vitamin B<sub>12</sub> content of turtle blood is 2.0, of chicken blood 1.0 and of turkey blood 1.5  $\mu\text{g./ml.}$  when the *L. leichmannii* activity due to thymidine is determined and subtracted.

#### SUMMARY

The vitamin B<sub>12</sub> content of blood from the human, dog, cotton rat, white rat, American albino mouse, Swiss albino mouse, cow, sheep, goat, pig, horse, rabbit,



turtle, chicken and turkey has been determined with *L. leichmannii* 4797 (ATCC). Values obtained varied from 0.5 to 10.1  $\mu\text{g}$ .  $\text{B}_{12}$ /ml. whole blood. The  $\text{B}_{12}$  content of rabbit blood was particularly high when compared to that of other species. The vitamin  $\text{B}_{12}$  activity of turtle, chicken and turkey blood was higher than that of other species tested with the exception of the rabbit and this is probably due to the fact that these animals have nucleated erythrocytes and a part of the *L. leichmannii* activity of such blood samples is due to thymidine. Autoclaving blood samples from the cotton rat, white rat, American albino mouse, Swiss albino mouse, calf, sheep, goat and rabbit at pH 12.0 destroyed all of the *L. leichmannii* activity; whereas, similar treatment of blood samples from the turtle, chicken and turkey destroyed only a portion of the vitamin  $\text{B}_{12}$  activity.

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# ROLE OF THE THYROID IN METABOLIC RESPONSES TO A COLD ENVIRONMENT<sup>1</sup>

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THE exposure of most warm blooded animals to a cold environment gives rise to a number of apparently related metabolic phenomena. An increase in metabolic rate as measured by oxygen consumption, takes place very rapidly and in the rat at a temperature just above freezing; the rate may reach a level two or three times that observed at the critical temperature of the animal (1, 2). When exposure is prolonged for more than a few days, hyperplasia of the thyroid gland has been observed repeatedly (3-5), and part of the increased rate of metabolism has been generally ascribed to increased activity of the thyroid gland. In relation to the known action of thyroxine, however, the rapid rise in metabolic rate and almost immediate increase of muscular activity has made it appear likely that whatever role the thyroid does play is of a slower, more gradual onset which might involve other than muscular tissues. The experiments reported in this paper extend previous observations on the relationship of the thyroid and pituitary to changes in metabolism which occur in a cold environment and indicate that while *increased* thyroid activity is not indispensable for an elevation in metabolic rate, part of the increase observed is dependent on the presence of thyroid hormone and is associated with the ability of the animal to survive.

## EXPERIMENTAL

1. *Oxygen Consumption of Normal Rats Exposed to Cold.* Male rats of the Wistar strain, weighing from 120 to 200 gm. were kept in individual metal cages and were fed a stock ration (Fox Breeder Cubes—Master Feeds) and water *ad libitum* at all times. Oxygen consumption was measured directly, using a method previously adopted by Ferguson and Sellers (6). Several estimations were obtained at a temperature of 30° C. and then the animals were placed in a cold room maintained at  $1.5^{\circ} \pm 1^{\circ}$  C. Additional values (expressed in cc. of oxygen consumed per m.<sup>2</sup> of body surface per minute) were obtained during a period of 3 months, first at daily and later at longer intervals. At least two estimations taken at the same hour on consecutive days were made after each interval. The measurements on one group were taken at the temperature of the cold room, these values being designated  $MR^{1.5}$ ,

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while measurements on another group were taken outside the cold room, the readings being designated  $MR^{30}$ .<sup>2</sup> Due to heat loss from the animals the temperature within the metabolism chambers of the group recorded in the cold room was actually somewhat higher than  $1.5^{\circ}\text{C}$ ., averaging approximately  $8^{\circ}\text{C}$ . The animals of the group recorded at  $30^{\circ}\text{C}$ . were kept at that temperature for 30 to 45 minutes prior to estimation of the oxygen consumption. When they were removed from the cold room they appeared to be restless for 10 to 15 minutes but during the measurement of oxygen consumption usually remained quiet. In every case values from animals which exhibited unduly large body movements were discarded. Colonic temperatures were recorded prior to every estimation of metabolic rate.

2. *Oxygen Consumption Under Anesthesia.* Estimations of oxygen consumption were carried out under sodium pentobarbital anesthesia on groups of rats weighing about 200 gm. maintained at  $1.5^{\circ}\text{C}$ . ( $MR^{1.5}$ ), on groups maintained at  $1.5^{\circ}\text{C}$ . but recorded at  $30^{\circ}\text{C}$ . ( $MR^{30}$ ), and on normal controls. The procedure consisted in administering 3 mg. of sodium pentobarbital per 100 gm. body weight intraperitoneally prior to the 30-minute period of 'stabilization' which preceded all metabolism readings.

3. *Oxygen Consumption of Rats Receiving Sodium Iodide.* Experiments similar to those described in section 1 were repeated, but instead of water the animals were allowed 0.05 per cent sodium iodide solution *ad libitum*.

4. *Oxygen Consumption of 'Athyroid' Rats With and Without Administration of Thyroxine.* When thyroidectomized rats were exposed to cold they survived at most for only a few days. In a group of thyroidectomized rats the  $MR^{1.5}$  was recorded once only, 4 to 5 hours after exposure to cold. A constant daily dose of 2.5  $\gamma$  or 5  $\gamma$  of thyroxine was administered subcutaneously to other groups of male rats (weight av. 200 gm.) rendered athyroid by operation, or by feeding propylthiouracil for 2 weeks before exposure, or by both. The administration of thyroxine was started 6 or 7 days prior to exposure and the  $MR^{1.5}$  and  $MR^{30}$  were recorded at 1- to 2-week intervals.

5. *Oxygen Consumption of 'Adapted' Rats Rendered Athyroid.* Animals which had been kept in the cold room for 6 to 8 weeks were subjected to thyroidectomy or to the administration of 0.02 per cent propylthiouracil incorporated in the diet. Rats so treated survived for 3 to 5 weeks, so that it was possible to obtain measurements of oxygen consumption at  $1.5^{\circ}\text{C}$ . and  $30^{\circ}\text{C}$ . in a satisfactory manner. These values were compared with those from thyroidectomized animals maintained at a normal room temperature.

6. *Estimation of Thyrotrophin.* Estimations of thyrotrophic activity were carried out on pituitaries of rats kept in the cold room for various periods of time (4-93 days). The method consisted of removing the glands immediately after sacrifice, separating the anterior from the posterior lobe and making a saline suspension of mixed anterior lobes. The equivalent of one-half or one gland was injected subcutaneously into each of 4 or 5 immature guinea pigs (150-200 gm.). This procedure

<sup>2</sup> The method of estimating oxygen consumption consisted of immersing plastic metabolism chambers in a water bath kept at a desired temperature and measuring the volume of oxygen at N.T.P. consumed. The superscripts  $1.5^{\circ}$ ,  $30^{\circ}$  etc., refer to the temperature of the water bath.

was repeated daily for four days. On the fifth day the guinea pigs were sacrificed and the thyroids were removed, blotted and weighed. The thyroid weights were compared with those obtained when purified thyrotrophin in graded doses was injected into guinea pigs of the same sex, weight and strain.

The effect of the administration of iodide on thyrotrophic activity of the pituitaries was measured by performing a similar experiment using rats given 0.05 per cent sodium iodide solution in lieu of water during the period of exposure to cold.

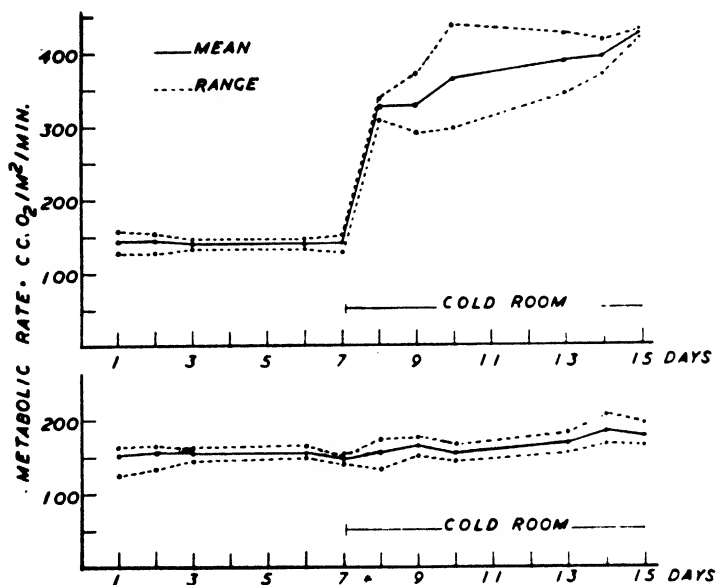


FIG. 1. OXYGEN CONSUMPTION of 8 male rats before and after exposure to cold. Measurements were made daily at 30° C. before exposure. After exposure to cold measurements on one group (4 rats, upper graph) were taken at the temperature of the cold room ( $MR^{1.5^\circ}$ ) while measurements on the other group (4 rats, lower graph) were taken outside the cold room at 30° C. ( $MR^{30^\circ}$ ).

In most experiments the thyroid and other tissues were placed in fixative immediately after death, and after appropriate preparation were examined histologically.

## RESULTS

When normal rats were exposed to cold the total metabolic rate rose rapidly, reaching a maximum of 2.7 times the resting values after 8 days. This is shown graphically (fig. 1, upper). In prolonged experiments (3 months) the  $MR^{1.5^\circ}$  varied considerably, there being some indication that it decreased somewhat towards the end of this period (fig. 2). The apparatus used for measurement of oxygen consumption was not designed for excessively high levels. More precise or more prolonged measurements will be needed to decide this question. The  $MR^{30^\circ}$  increased very

gradually, reaching a value 17 per cent higher than before in 8 days (fig. 1, lower). Usually values from 30 to 50 per cent higher than normal were ultimately reached, but after 2 weeks of exposure little further increase took place. During the 3-month period of observation no decline was observed (fig. 2). The colonic temperature of the rats in the cold room (average of 8, first 6 days exposure) was  $37.7^{\circ}\text{C}$ . compared to a temperature of  $37.1^{\circ}\text{C}$ . (average) obtained from the same rats for 5 days prior to exposure. The difference is significant ( $P < .01$ ).

Under sodium pentobarbital anesthesia both the  $MR^{1.5^{\circ}}$  and  $MR^{30^{\circ}}$  were considerably lower than the values obtained previously. In the animals measured at  $1.5^{\circ}\text{C}$ . the oxygen consumption was reduced to about 50 per cent of the figure obtained without anesthesia and the colonic temperature fell by about  $6^{\circ}\text{C}$ . during the period of anesthesia, indicating that heat loss was exceeding heat production. In control animals from a normal environment a pronounced fall in  $MR^{30^{\circ}}$  also took

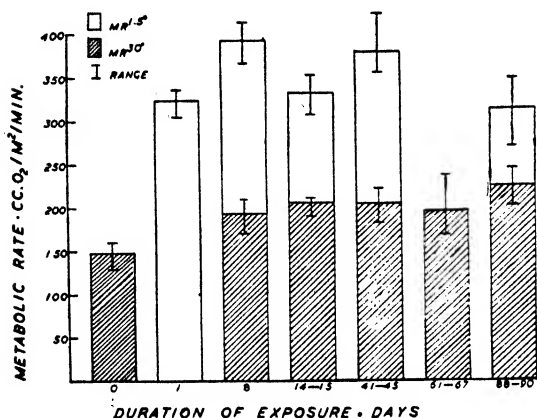


FIG. 2. OXYGEN CONSUMPTION ( $MR^{30^{\circ}}$  and  $MR^{1.5^{\circ}}$ ) of groups of rats (4-8 rats in each group) after exposure to cold for varying length of time.

place. The absolute decrease was much less than that of the animals measured at  $1.5^{\circ}\text{C}$ . and less than that of the 'cold' animals measured at  $30^{\circ}\text{C}$ . In proportion to the figures obtained before anesthesia the decrease in oxygen consumption under anesthesia of normal controls and animals kept in the cold room but measured at  $30^{\circ}\text{C}$ . was similar (17-21% decrease, table 1). However, the absolute values from the anesthetized normal group were significantly lower than those of the rats exposed to cold whether the estimations were made at  $1.5^{\circ}$  or  $30^{\circ}\text{C}$ .

Table 2 summarizes the results of  $MR^{30^{\circ}}$  estimations on groups of male rats kept at a temperature of  $1.5^{\circ}\text{C}$ . and given 0.05 per cent sodium iodide solution instead of water. No significant differences in metabolic rate exist between control and experimental groups. However, the thyroid weights of the iodide-treated group are significantly lower than those of the controls (table 6). Figure 3 a, b, illustrates the typical histological ( $\times 560$ ) appearance of the two groups after 2 weeks' exposure. It is evident that in the iodide-treated group signs of activity are less marked.

The effect of thyroidectomy on the changes in metabolic rate is complicated

by the fact that when thyroidectomized animals are exposed to a temperature of  $1.5^{\circ}\text{C}$ . even several weeks after the operation, they survive only a few days. Animals which received propylthiouracil for 2 weeks or more reacted in the same way. However, when recordings were made a few (4-5) hours after exposure to a temperature of  $1.5^{\circ}\text{C}$ . a pronounced rise of  $MR^{1.5}$  did occur.

By giving a small constant dose of thyroxine daily, thyroidectomized animals could be maintained in the cold indefinitely and any possibility of increased production of thyroid hormone due to the experimental conditions obviated. In thyroidectomized, propylthiouracil-fed animals (average weight 200 gm.) given 2.5  $\gamma$  or 5  $\gamma$

TABLE 1. EFFECT OF ANESTHESIA (SODIUM PENTOBARBITAL) ON  $MR^{30^{\circ}}$  OF NORMAL- AND COLD-ROOM RATS (WT. 200 GM.)

NO. IN GROUP	SEX	DURATION IN COLD	$MR^{30^{\circ}}$				AVERAGE DECREASE
			WITHOUT ANESTHESIA		WITH ANESTHESIA		
			Av.	Range	Av.	Range	
			<i>days</i>				
8	F	0	117	107-133	95	77-120	18.8
4	M	0	143	127-158	117	103-130	18.2
8	F	45-57	175	157-185	137	119-150	21.6
7	M	35-48	176	157-200	145	138-154	17.1

TABLE 2. OXYGEN CONSUMPTION ( $MR^{30^{\circ}}$ ) OF MALE RATS IN COLD ENVIRONMENT RECEIVING NaI

NO. IN GROUP	AV. BODY WT.	DURATION IN COLD	NaI	$MR^{30^{\circ}}$	
				Av.	Range
	gms.	days	%		
8	201	14-10	0.05	187	168-200
8	215	14-10	0	191	162-206
4	130	20	0.05	195	184-218
4	153	20	0	204	188-228

of thyroxine daily, an increase in  $MR^{1.5}$  and in  $MR^{30}$  took place. In both cases the increases simulated those of normal animals in time-relation and degree.<sup>3</sup> The same finding was observed in thyroidectomized animals given 5  $\gamma$  of thyroxine but not receiving propylthiouracil. These results are presented in table 3. On removal from the cold room a return to the previous rate occurred within 3 to 4 days (fig. 4).

Under anesthesia the oxygen consumption values of such rats fell (table 4) by about the same percentage as did anesthetized, thyroidectomized animals kept at room temperature. The absolute values obtained from the 'cold' rats were almost twice those of the animals maintained at room temperature.

In animals accustomed to the cold environment ('adapted') surgical thyroidec-

<sup>3</sup> Expressed as percentage increase. The absolute figures were not so high as most readings from normal rats kept in an environment of  $1.5^{\circ}\text{C}$ .

tomy or propylthiouracil administration no longer caused death within several days although life was prolonged only by some 3 to 5 weeks. Oxygen consumption ( $MR^{30}$ ) measured two weeks after surgical or medical 'thyroidectomy' was of the same low order as that of athyroid rats maintained at room temperature (table 5). The actual figures obtained were on the average 10 per cent higher than in the athyroid group at room temperature. With the number of animals and variation in readings no significance could be assigned to this difference ( $P = 0.1-0.2$ ). The  $MR^{1.5}$  (4 animals only) showed that a well marked rise in total heat production occurred but the values were less than for normal controls or for thyroidectomized animals given a small fixed daily dose of thyroxine.

When pituitary material from rats maintained in the cold environment was injected daily for 4 days into immature guinea pigs, the weights of the thyroid glands of the recipient animals were greater than those of guinea pigs which received equivalent amounts of pituitary from rats kept at room temperature. This change was

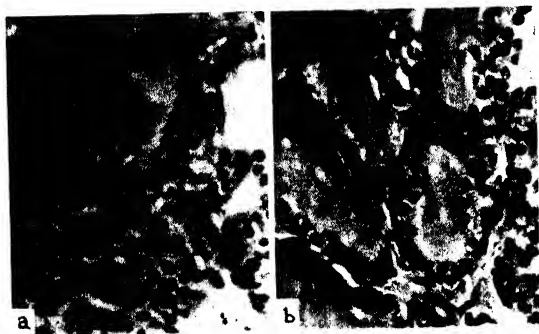


FIG. 3. PHOTOMICROGRAPHS showing effect of iodide on histological appearance of thyroid glands of rats exposed to cold for 14 to 17 days. *a*: Gland from control rat without iodide. *b*: Gland from rat receiving 0.05 per cent sodium iodide in drinking water (H & E  $\times 200$ ).

not apparent after the first period of exposure tested (4-10 days) but was true of the last three periods, namely 14 to 17 days, 29 to 33 days and 90 to 93 days (table 6). The histological appearance of the guinea pig thyroids was consistent with these observations, the degree of hyperplasia increasing with the weight of the gland.

Pituitaries from rats which had been given 0.05 per cent sodium iodide solution while exposed to cold had approximately the same thyrotrophic activity as control animals in the same environment.

#### DISCUSSION

The increase in oxygen consumption which takes place when rats are exposed to a temperature of  $1.5^{\circ}\text{C}$ . is rapid in onset and striking in degree. In rats, involuntary muscle movements are not easily seen, and voluntary activity may not be increased in any obvious fashion. Nevertheless, the increased oxygen consumption has been shown to be associated with muscular activity. The part which non-muscular tissue may play in increasing heat production is difficult to demonstrate, and has been approached in several ways. Collip and Billingsley (7) observed that if rats kept at  $-4$  or  $-5^{\circ}\text{C}$ . were suddenly exposed to a temperature of  $28^{\circ}\text{C}$ . they appeared to be 'uncomfortable' and moved about to an unusual extent. They ob-

served that this did not occur at 16° C., so oxygen consumption measurements were made at this temperature in order to rule out increased muscle activity as a factor in the measurements. In the present study it was observed that after 15 to 30 minutes of exposure to 30° C. the 'cold' animals became quiet and seemed comfortable enough. The gradual increase in oxygen consumption values obtained at 30° C., over a period of 8 to 10 days indicates that some change takes place during this period. The factors responsible must differ in some respects from those producing the immediate increase observed when recordings are made at 1.5° C.

TABLE 3. METABOLIC RESPONSE TO COLD OF THYROIDECTOMIZED MALE RATS (WT. 200 GM.) RECEIVING A CONSTANT DOSE OF THYROXINE

NO. IN GROUP	THYROXINE	PROPYL-THIOURACIL IN FOOD	MR <sup>30°</sup>				INCREASE
			BEFORE EXPOSURE		2-5 WEEKS DURING EXPOSURE		
			Av.	Range	Av.	Range	
	γ/day	%					%
8	5	0	120 <sup>1</sup>	118-128	167	154-178	39
7	2.5	0.02	108 <sup>1</sup>	101-116	166	147-209	54
4	5	0.02	136	128-156	152 <sup>2</sup>	144-166	12

<sup>1</sup> Four rats only.    <sup>2</sup> Three rats only.

TABLE 4. EFFECT OF ANESTHESIA (SODIUM PENTOBARBITAL) ON MR<sup>30°</sup> OF THYROIDECTOMIZED MALE RATS RECEIVING A CONSTANT DOSE OF THYROXINE

NO. IN GROUP	PROPYL-THIOURACIL IN DIET	THYROXINE	DURATION IN COLD	MR <sup>30°</sup>		
				Without anesthesia	With anesthesia	Av. decrease
	%	γ/day	days			%
3	0	5	39-40	166	134	19.3
3	0.02	5	21-23	155	113	27.1
3	0.02	2.5	0	91	68	25.2

In order to decrease muscular activity still further, resort to general anesthesia was made and it could be demonstrated that under anesthesia, both at 1.5° C. and at 30° C. the oxygen consumption was higher than in controls kept at room temperature. It would be fallacious to state that this has eliminated the muscular element, nor can it be assumed that in proportion to previous metabolic rates, muscular activity of the three groups ('cold' rats measured at 1.5° C., 'cold' rats measured at 30° C., controls measured at 30° C.) decreased equally. The effect of barbiturate anesthesia on muscular and glandular tissue-respiration and metabolism may not be similar, and may vary with the state of the tissue at the time. It seems even more difficult to accept Collip and Billingsley's suggestion that when no visible signs of discomfort are present an increase in muscle tone does not exist. Myographic studies were not carried out either by Collip and Billingsley or by us.

The concurrent, gradual increase in MR<sup>30°</sup>, the increase in weight and in appear-



ance of 'activity' of the thyroid, and the increase in thyrotrophic activity of the anterior pituitary suggest the possibility that these changes are causally related with one another. That the relationship of these phenomena is not a satisfactory explanation of the increased  $MR^{30^\circ}$  is advanced in the following paragraphs.

Starr and Roskelley (5), and Lesser, Winzler and Michaelson (8) have reported that administration of iodide reduced thyroid weight increase and hyperplasia in rats after exposure to cold and this observation has been confirmed in the present study. Although iodide decreased hyperplasia it did not affect the elevation in  $MR^{30^\circ}$ .

A daily injection of a constant dose of thyroxine (2.5  $\gamma$ ) to thyroidectomized rats fed propylthiouracil was not sufficient to keep the metabolic rate within the

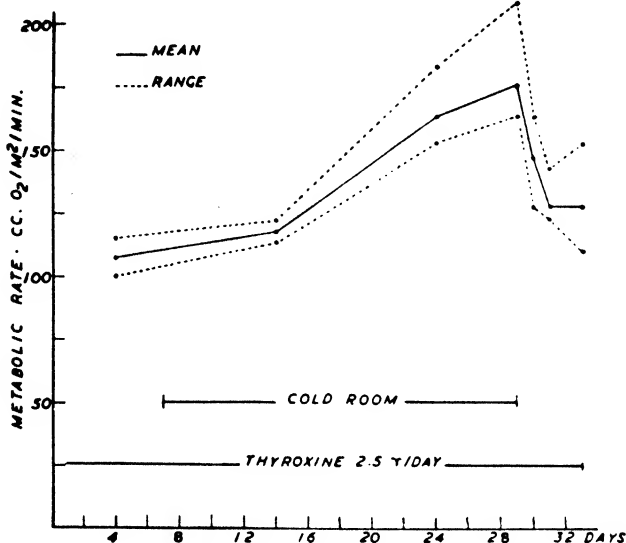


FIG. 4. METABOLIC rate ( $MR^{30^\circ}$ ) of 4 thyroidectomized rats on propylthiouracil and thyroxine (2.5  $\gamma$ /rat/day) before, during and after exposure to cold. Thyroxine was started one week before exposure to cold.

normal range. In spite of the fact that these animals received a constant, small dose of thyroxine, when they were exposed to cold the  $MR^{30^\circ}$  rose gradually as it does in normal animals. Although the level attained was not so high as in normal animals, the percentage increase was actually higher (average, 54%). When a constant daily dose of 5  $\gamma$  of thyroxine was injected into thyroidectomized rats with or without concurrent feeding of propylthiouracil, the  $MR^{30^\circ}$  level attained was not appreciably different from that of animals receiving the lower dosage.

It is interesting that athyroid rats with or without propylthiouracil feeding do not survive for more than a few days if the environmental temperature change is rapid. MacBeth and Noble (9) decreased the environmental temperature in stages over a period of several weeks and reported that  $MR^{30^\circ}$  values increased. In our experiments animals which had lived in a cold room for 6 to 12 weeks and were

thyroidectomized or given propylthiouracil then were able to withstand the low temperature for a period of 3 to 5 weeks. The rate of oxygen consumption (4 rats receiving propylthiouracil) at  $1.5^{\circ}\text{C}$ . was increased to double the average value obtained in athyroid rats at  $30^{\circ}\text{C}$ ., again showing that increased metabolism can take

TABLE 5. OXYGEN CONSUMPTION ( $MR^{30^{\circ}}$ ) OF 'ATHYROID' RATS IN COLD AND AT ROOM TEMPERATURE

GROUP	ENVIRONMENT	NO. OF RATS	$MR^{30^{\circ}}$	
			Mean $\pm$ S.D.	$P^2$
Propylthiouracil <sup>1</sup> .....	Cold	4	110.8 $\pm$ 8.5	$>.2$
Thyroidectomy <sup>1</sup> .....	Cold	6	113.2 $\pm$ 14.9	$>.1$
Thyroidectomy .....	Normal	7	102.3 $\pm$ 9.9	

<sup>1</sup> Made 'athyroid' after 'adaptation' to cold for 38 to 78 days. <sup>2</sup> Compared to thyroidectomized rats in normal temperature.

TABLE 6. EFFECT OF EXPOSURE TO COLD FOR VARYING PERIODS ON THYROID WEIGHT AND THYROTROPIC CONTENT OF PITUITARY OF MALE RATS

NO. IN GROUP	AV. BODY WT.	DURATION IN COLD	THYROID WT.	THYROTROPIC ASSAYS		
				NO. OF GUINEA PIGS	AV. THYROID WT.	ESTIMATED TSH <sup>1</sup>
	gm.	days	mg/100 gm. B.W. $\pm$ S.D.		mg/100 gm. B.W.	mg/gland
8	146	4-10	12.1 $\pm$ 1.2	4	26.8	0.059
8	145	0	8.5 $\pm$ 0.8	4	25.5	0.052
8	209	14-17	14.0 $\pm$ 2.4	4	42.2	$>0.125$
8 <sup>2</sup>	210	14-17	11.6 $\pm$ 1.2	4	41.7	$>0.125$
8	226	0	8.4 $\pm$ 0.9	4	34.7	0.084
12	174	20-33	13.1 $\pm$ 1.3	6	43.5	$>0.125$
12	168	0	7.1 $\pm$ 1.1	6	32.5	0.072
6	260	90-93	11.4 $\pm$ 2.0	3	41.4	$>0.125$
6	267	0	8.1 $\pm$ 3.0	3	30.3	0.061
20	198	22-26	11.4 $\pm$ 2.4	5	34.1	0.090
20 <sup>3</sup>	246	0	8.5 $\pm$ 1.6	5	30.6	0.064

<sup>1</sup> Estimated TSH compared to standard preparation of thyrotrophic material supplied through the kindness of Dr. D. A. McGinty, Parke, Davis and Company, Detroit, Michigan.

<sup>2</sup> Drinking water contained 0.05 per cent sodium iodide. <sup>3</sup> Control rats of same age, but greater body weight.

place in the absence of the thyroid. However, the readings were much lower than the  $MR^{1.5^{\circ}}$  of normal animals. The  $MR^{30^{\circ}}$  readings were on the average slightly higher (about 10%) than values usually obtained in athyroid rats at  $30^{\circ}\text{C}$ . While this would agree with the finding of MacBeth and Noble (9) cited above, that an increase in the  $MR^{30^{\circ}}$  can take place in the absence of the thyroid, the observed

difference is not statistically significant and in any case the level of oxygen consumption was 'hypothyroid', being consistently below the range found in normal rats kept at room temperature.

It appears, therefore, that with thyroidectomized rats survival at 1 to 2° C. may be prolonged to some extent by gradually decreasing the temperature (9) or by delaying removal of the thyroid until the animals become accustomed to living at a temperature just above freezing. It seems possible that prolonged action of existing thyroid hormone, or possibly extra-thyroidal formation of traces of the hormone may account for the prolongation of life by several weeks. A level of thyroxine administration which is insufficient to maintain a normal metabolic rate (2.5  $\gamma$  per day) makes survival possible and permits an increase of  $MR^{30^\circ}$  of the same degree (based on oxygen consumption before exposure to cold) and approaching the same level as in the normal animal. It has been pointed out that the effect with 5  $\gamma$  of thyroxine per day is no greater than with 2.5  $\gamma$ . Nevertheless the absolute values obtained both before and after exposure were less than those found in normal animals. This evidence makes us believe that the increase in  $MR^{30^\circ}$  which normally occurs on exposure to cold is dependent on the presence of some thyroid hormone.

It may be added that in these experiments no important differences were noticed in the reactions of animals after thyroidectomy and after propylthiouracil feeding for 2 to 3 weeks.

The amount of thyrotrophic hormone in the pituitaries of animals exposed to cold is increased significantly. This increase was noted after two weeks and was maintained for the period of observation (13 weeks). Within the limits of sensitivity of the method of assay employed no change in activity was observed after the first two weeks of exposure, although the activity of one week was lower and did not show a measurable increase over normal animals. These findings are referable to the activity of the entire anterior pituitary but not necessarily to the activity per mg. of pituitary tissue. The thyrotrophic activity of rats which received sodium iodide and were exposed to cold was of the same order as non-iodide treated animals, that is, while iodide caused a decrease in weight and appearance of 'activity' of the thyroid, no decrease in thyrotrophin in the pituitary was demonstrated. There is experimental evidence (10) that when active thyrotrophic extracts are injected in animals receiving iodide, the effect on the thyroid is less than expected. The iodide might be termed 'antithyrotrophic.' This suggests that the effect of iodide reported here is not due to preventing the release of thyrotrophin but to a more direct action, perhaps by enabling the gland to synthesize thyroid hormone more effectively or perhaps by inactivating the thyrotrophic hormone in some way.

#### SUMMARY AND CONCLUSIONS

When adult rats were exposed to a low environmental temperature ( $1.5^\circ \pm 1^\circ$  C.) an increase in  $MR^{1.5^\circ}$  (total metabolic rate measured at  $1.5^\circ$  C.) took place immediately and an increase in  $MR^{30^\circ}$  (metabolic rate measured at  $30^\circ$  C.) occurred gradually reaching a maximum in about two weeks. The high levels of  $MR^{1.5^\circ}$  and  $MR^{30^\circ}$  were maintained for a period of at least three months, the period of observation.

The highest average values of  $MR^{1.5^\circ}$  and  $MR^{30^\circ}$  were 2.7 and 1.5 times the values obtained before exposure to cold.

$MR^{1.5^\circ}$  was greatly reduced when the oxygen consumption was measured under sodium pentobarbital anesthesia, suggesting that a large part of the increase could be attributed to muscular activity.

Increased muscular activity (movement) did not appear to be responsible for the raised  $MR^{30^\circ}$  as anesthesia did not cause a reduction in  $MR^{30^\circ}$  to as low a level as, or by a greater percentage than in normal controls.

In 'athyroid' rats (thyroidectomy or propylthiouracil) which received a constant dose of 2.5 to 5  $\gamma$  of thyroxine daily a marked increase in  $MR^{30^\circ}$  occurred during exposure to cold. Thus the elevation of metabolic rate in cold is not dependent on increased activity of the thyroid. Although administration of sodium iodide decreased the degree of thyroid hyperplasia in cold it did not affect the elevated  $MR^{30^\circ}$ .

Thyroidectomized rats failed to survive for more than a few days exposure to  $1.5^\circ\text{C}$ ., so that prolonged observation was impossible. Rats acclimatized to this temperature lived long enough after surgical or 'medical' thyroidectomy for studies on metabolic rate to be carried out. The  $MR^{1.5^\circ}$  was elevated but the  $MR^{30^\circ}$  of these athyroid rats approached the low values observed in athyroid rats living at room temperature.

An increase in thyrotrophic activity of the pituitaries was found after two weeks of exposure to cold and this increase was sustained during a period of three months of observation. The enhanced thyrotrophic activity was not affected by the administration of sodium iodide.

It is postulated that the increase in metabolic rate ( $MR^{30^\circ}$ ) in animals after exposure to cold does not depend on a hyperthyroid state but does depend on the presence of thyroid hormone and that this increase in metabolic rate is associated with the ability of the animal to survive.

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# EFFECT OF ENVIRONMENTAL TEMPERATURE AND STARVATION ON ADRENAL GLANDS OF THE WHITE-FOOTED MOUSE

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THE stimulating effect of low temperature upon the adrenal cortex and medulla is well known. A number of investigators have also noted the effect of starvation and underfeeding upon the adrenal gland. Marrian (1) produced adrenal hypertrophy in pigeons by underfeeding and suggests that hypertrophic changes occurred mainly in the medulla. Mulinos *et al.* (2) found that the adrenals of female rats starved for 7 days increased 38 per cent in weight, while those of males starved for the same length of time increased 22 per cent in weight. A proportional rise in vitamin C content with increasing adrenal weight was believed to represent true physiologic growth rather than a condition of hyperemia or edema. More recently, D'Angelo, Gordon and Charpper (3, 4) have observed that in female guinea pigs starved to 40 per cent body weight loss by complete food deprivation the adrenal glands underwent absolute hypertrophy (60 per cent) with a commensurate increase in solid contents.

The following observations were made incident to a study of the resistance to starvation at different temperatures of wild deer mice having previously different thermal histories. The possible synergistic action of temperature and starvation upon adrenal enlargement should not be overlooked although the evidence from this study is insufficient to warrant any conclusions as to such an effect.

## METHODS

Specimens of the white-footed mouse, *Peromyscus leucopus noveboracensis* (Fischer), used in this study were taken from wild populations in the vicinity of Champaign-Urbana, Illinois from September 1947 to March 1949.

Survival times were determined over a range of air temperatures from  $-33^{\circ}\text{C}$ . to  $+50^{\circ}\text{C}$ . Various constant temperature rooms, incubators, and refrigerators were used in which air temperatures were held constant within  $\pm 1^{\circ}\text{C}$ . The percentage relative humidity in the different chambers was uncontrolled and varied inversely with the air temperature. In survival tests at different temperatures individual mice were confined separately and deprived of all food and water.

The adrenal glands were removed and weighed shortly after death had occurred.

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Weighings were made upon a chainomatic analytical balance to the nearest milligram. All superfluous fat around each gland was removed before weighing. Adrenal weights which provided the control values were taken on snap-trapped animals within a few hours of capture and therefore were considered to be affected only by those temperatures at which they had been living and not affected by starvation.

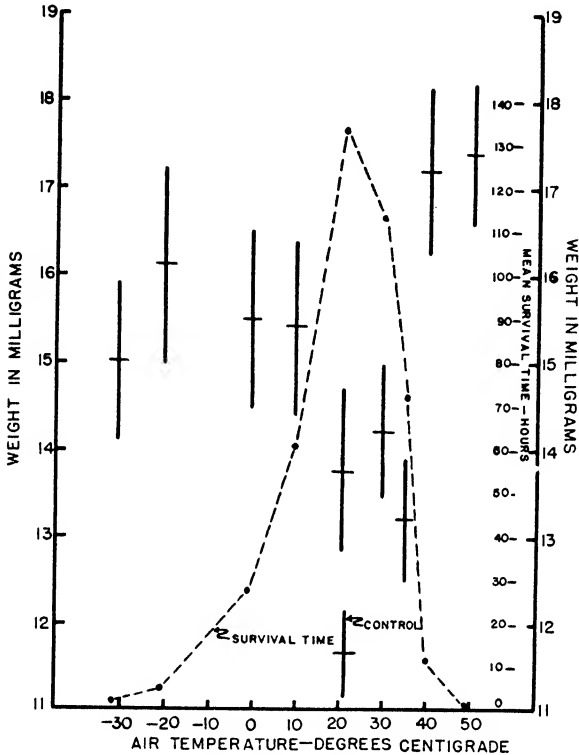


Fig. 1. RELATIONSHIP between air temperature and adrenal weights of *P. l. noveboracensis* surviving at different air temperatures without food or water.

#### RESULTS AND DISCUSSION

No consistent differences in adrenal weights of animals having previously different thermal histories could be found at different survival temperatures. For this reason all of the adrenal weights for animals surviving at a given temperature were grouped together.

A definite correlation between adrenal weights and length of survival at different air temperatures is not evident (table 1). A general trend toward increasing adrenal weights with increasingly low air temperature is indicated, however (fig. 1).

The mean adrenal weights of all animals undergoing survival tests differed significantly from control values. Control animals were trapped in September during

a period in which air temperatures ranged from 17° to 25°C. Temperatures during the previous month were warmer by several degrees.

Statistical comparison has been made according to the method developed by Dice (5). The means are represented by short cross-bars with one standard error of the mean, plus or minus, represented by the heavy vertical bars (fig. 1). If the ranges about the mean so laid out overlap (comparison being easily made with a straight edge) it may usually be assumed that the differences between the means are not significant while if they do not it may usually be assumed that these differences are statistically significant.

At 40° and 50°C. hyperemia was very evident and probably accounted for most of the significant increase in weight. Most of the adrenal glands from animals surviving at these temperatures were visibly engorged with blood, the majority of them

TABLE 1. COMPARISON OF COMBINED ADRENAL WEIGHTS OF *P. L. NOVEBORACENSIS* AFTER DEATH AT DIFFERENT AIR TEMPERATURES IN ABSENCE OF FOOD AND WATER

MEAN AIR TEMP.	NO. OF ANIMALS	MEAN WEIGHT OF ADRENALS <sup>1</sup>	STANDARD DEVIATION	INCREASE OVER CONTROLS	MEAN SURVIVAL TIME
°C.		mg.		%	hr.
50	20	17.39 ± .79	3.51	49.27	0.8
40	20	17.22 ± .94	4.20	47.81	11.6
35	22	13.19 ± .68	3.20	13.22	71.7
29 to 30	21	14.21 ± .74	3.38	21.97	113.5
20 to 21	21	13.73 ± .96	4.40	17.85	133.9
8 to 11	21	15.40 ± .97	4.42	32.19	60.8
-1 to 0	20	15.49 ± .99	4.44	32.96	27.4
-21 to -20	21	16.10 ± .12	5.15	38.19	4.8
-33 to -30	24	14.99 ± .86	4.21	28.67	1.7
Controls	22	11.65 ± .52	2.46		
17 to 25					

<sup>1</sup> Plus or minus one standard error of the mean.

having a dark reddish-black appearance. Some hyperemia was noticeable at extreme low temperatures (-20° to -33°C.) but was not so marked as at the extreme high temperatures. There was little or no evidence of hyperemia at other temperatures.

Increases in adrenal weight over control values ranged from 13 to 49 per cent. The percentage increase in weight was the least at intermediate and moderately high temperatures. No apparent correlation between length of survival and percentage increase in weight over control values is evident (table 1). Within the range of temperatures where hyperemia was not evident adrenal weights increased most at low temperatures and this weight increase apparently represents true physiologic growth. Starvation is considered to be the most probable cause for the significant increase in weight at intermediate temperatures, while it is possible that the additional weight increases at low temperatures may have been brought about by further stimulation of the adrenals to greater activity by the low temperature with an attendant increase in size of the glands.

The hypertrophic changes observed in this and other studies may involve either

the medulla or cortex, but there is no definite agreement on this point. Observed increase in vitamin C content resulting from starvation (Mulinos *et al.*, 2) are suggestive in connection with the experiments of Dugal and Therien (6) and Therien and Dugal (7) who found that a large increase in the ascorbic acid content of the tissues of the white rat occurs after prolonged exposure and acclimation to cold ( $-4^{\circ}$  to  $4^{\circ}\text{C}$ ). A decrease in ascorbic acid content of the tissues was found in rats unable to adjust themselves to a cold environment. Long and Fry (8) found that adrenaline and exposure to cold cause a definite decrease in the ascorbic acid content of the adrenals of rats, while Tyslowitz (9) reports an increase in vitamin C content of rat adrenals upon administration of suitable pituitary extracts.

If increased adrenal weights of white-footed mice occurring after exposure to different air temperatures in the absence of food and water represent true physiologic growth and are accompanied by corresponding increases in vitamin C content, then it is possible that adrenal enlargement brought about by starvation or by temperature acting via the pituitary might increase resistance to low temperature.

#### SUMMARY

Hypertrophic changes occurred in the adrenals of white-footed mice, *Peromyscus leucopus noveboracensis* (Fischer), subjected to starvation at different air temperatures. Hyperemia accounted for most of the glandular weight increases at  $40^{\circ}$  to  $50^{\circ}\text{C}$ . and at  $-20^{\circ}$  to  $-33^{\circ}\text{C}$ ., while starvation seemed to be the most probable cause for adrenal enlargement at intermediate temperatures. Within the range of temperatures where no hyperemia was evident low temperatures brought about the greatest increase in adrenal weight over control values.

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# CHANGES IN CIRCULATING EOSINOPHILES AND ADRENAL ASCORBIC ACID CONCENTRATION AFTER AGENTS ALTERING BLOOD SUGAR LEVELS AND AFTER SURGICAL CONDITIONS

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SINCE the report by Hills *et al.* (1) that the number of circulating eosinophiles were reduced in patients with adequate adrenocortical function following administration of ACTH, an induced eosinopenia has been reported in patients and animals treated with ACTH, epinephrine, compounds E or F, and also women in labor and parturition (2-4). The observed depression of the number of circulating eosinophiles has been attributed to increased adrenocortical function as a result of direct stimulation of the glands or the stress phenomenon. Although the eosinopenic response has been attributed to increased elaboration of the 11-oxysteroids of the adrenal cortex but not to DCA (2), this has not been actually demonstrated nor has an eosinophilia been observed in Addisonian patients or adrenalectomized animals.

It was considered that the problem of the relation of the number of circulating eosinophiles to adrenocortical function would be further elucidated by investigation of the effect of certain experimental conditions, namely, those which might affect the activity of the adrenal cortex in the elaboration of its 11-oxysteroids or 'sugar hormones,' or the function of the adrenal gland as a whole. It is the purpose of this paper to report the effect of certain surgical procedures and agents which alter the blood glucose concentration upon the number of circulating eosinophiles and the functional status of the adrenal cortex (as measured by ascorbic acid concentration).

## METHODS

Male Wistar rats weighing 200 to 250 gm. were used. They were fed a chow biscuit ration supplemented with greens once weekly and tap water *ad libitum* (the drinking solution for the adrenalectomized rats was one per cent NaCl). The eosinophile counts were enumerated by the direct method of Randolph (5) on tail blood drawn immediately before and after the experimental procedures. The difference between the counts enumerated in each rat before and after the specific procedure was calculated and the mean difference of the group was tested for statistical significance. The adrenal ascorbic acid concentration was determined by the method of Roe and Keuthner (6) after the adrenals had been cleared of all extraneous tissue and weighed on a Roller-Smith balance to the nearest 0.1 mg. Blood glucose levels

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were determined by the method of Somogyi-Schaffer-Hartmann (7) on blood obtained by cardiac puncture with the rats under Evipal (*n*-methylcyclo-hexenyl-methyl barbituric acid) anesthesia. The blood glucose values of the controls obtained by this method were slightly higher than those usually reported. Since the rats did not appear emotionally disturbed, it is possible that the administration of the anesthetic induced a small rise in blood sugar.

The groups of rats used in this investigation and the results are reported according to the general procedures which were followed in the design of the experiments:

*Normal Intact.* (a) *ad libitum* fed; (b) after an over-night (approximately 16 hours) fast; and (c) after a 48-hour fast;

*Effect of Epinephrine.* A 0.02 per cent adrenaline solution in physiological saline was injected subcutaneously at a dosage level of 0.02 mg/100 gm. body weight (0.1 ml.). After obtaining the first tail blood sample for the enumeration of the eosinophiles, some of the groups of rats were given a single injection or a series of 3 successive injections of epinephrine at hourly intervals. The animals were killed at different time intervals after the epinephrine injections: (d) *ad libitum* fed and 180 minutes after the last of 3 successive injections at hourly intervals; overnight fasted and (e) 60 minutes, and (f) 180 minutes after a single injection of epinephrine; (g) adrenalectomized and 60 minutes after a single injection; and 180 minutes after the last of 3 successive injections at hourly intervals in the following groups: (h) adrenalectomized; (i) splenectomized; (j) adrenalectomized-splenectomized; and (k) sham adrenalectomized-sham splenectomized. All of the operated rats used in this series were 8 to 9 days post-surgery before the epinephrine administrations.

*Effect of Glucose per Os.* All the rats in this series were fasted overnight and then 2 ml. of a 50 per cent glucose solution was administered by means of a narrow plastic, flexible stomach tube while the rats were under light (Evipal) anesthesia. Tail blood for the enumeration of the number of circulating eosinophiles was drawn immediately before the glucose administration. The animals were killed at the following time-intervals: (l) 30 minutes; (m) 60 minutes; and (n) 180 minutes after the glucose *per os*.

*Effect of Insulin.* The insulin administered was prepared by accurately diluting the Lilly preparation Iletin with 0.85 per cent saline. Immediately after obtaining the first tail blood sample for the enumeration of circulating eosinophiles the rats were injected subcutaneously with 0.5 u (0.6 ml.) of this freshly prepared insulin solution. All the rats in this series were fasted overnight before the injections, and they were killed at the following time-intervals after insulin administration: (o) 30 minutes; (p) 60 minutes; (q) 120 minutes; and (r) 180 minutes after insulin.

*Effect of Surgery.* Eosinophiles were enumerated before and 8 to 9 days after surgery in the following groups of rats: (s) adrenalectomized; (t) splenectomized; (u) adrenalectomized-splenectomized; (v) sham splenectomized; and (w) sham adrenalectomized-sham splenectomized.

*Control-Treated.* Two groups of overnight-fasted intact rats in which the number of circulating eosinophiles were enumerated before and the animals killed 30 minutes after (x) the subcutaneous injection of 0.6 ml. of 0.85 per cent saline; and (y) after the administration of 2 ml. of distilled water by stomach tube.

The dosage of adrenaline used in this study has been established as a stimulus

which effectively increased adrenocortical activity (8, 9). The glucose *per os* and the insulin injections were used as 2 other means of influencing adrenocortical function since it has been reported that these agents affect the secretory activity of the adrenal cortex (10-12). It is well known that the 3 agents also rapidly and markedly affect the blood glucose level. It has been shown that approximately 180 minutes after ACTH or epinephrine there is a maximum depletion of adrenal ascorbic acid concentration (8) and a maximum reduction in the number of eosinophiles per cu. mm. of blood (2). The time intervals after the administration of adrenaline, insulin or glucose were followed in this investigation in order to determine whether the relation between the number of circulating eosinophiles and the functional status of the adrenal cortex might be influenced by a time factor.

The overnight-fasted rat was used in these experiments in order to have groups of rats which were uniformly 'basal' at the time of the administration of the several agents used and thereby eliminate, to some extent, the variability between rats in their response to these agents which might exist due to differences in feeding habits, or quantity of food undergoing intestinal absorption at the time of the experimental procedures.

#### RESULTS

A statistically significant decrease in the number of eosinophiles per cu. mm. of blood was induced by the experimental procedure in the following groups of rats (table 1): overnight-fasted normal intact, *group b*; 180 minutes after a single injection of epinephrine, *group f*; and 180 minutes after 3 successive hourly injections of epinephrine in *groups d, i, and k*; 180 minutes after glucose *per os*, *group n*, and insulin injection *group r*; and a probably significant decrease after sham adrenalectomy-splenectomy, *group w*. Inspection of these data shows that a statistically significant decrease in the number of circulating eosinophiles had been induced 1) after the administration of epinephrine, insulin or glucose *per os*, 2) only when the adrenal glands were *in situ*, and 3) the effect of these agents on depressing the number of eosinophiles per cu. mm. of blood was evident only in those groups in which 180 minutes had elapsed since the administration of these agents.

A statistically significant increase in the number of eosinophiles per cu. mm. of blood had been induced in the following experimental groups: after splenectomy, *group t*; after adrenalectomy-splenectomy, *group u*; after epinephrine injection in adrenalectomized rats, *group h*; and 60 minutes after insulin injection in intact rats, *group p*. Inspection of these results (table 1) shows that a significant increase in the number of circulating eosinophiles was induced in those groups in which the spleen was absent, or when epinephrine could not affect adrenocortical activity.

The effect of epinephrine, insulin, glucose *per os* or fasting on the adrenal ascorbic acid concentration and its relation to the changes in the number of circulating eosinophiles after the administration of these agents is shown in table 2. The hypoglycemic action of the insulin injection is evident in the 4 groups of rats so treated. However, a significant depletion of the adrenal ascorbic acid concentration was determined only in the 2 groups killed 120 and 180 minutes after the administration of 0.5 U of insulin. This adrenal ascorbic acid depletion was associated with a sta-

tistically significant decrease in the number of eosinophiles per cu. mm. of blood only in the group of rats 180 minutes after the administration of insulin. It is noteworthy that a significant increase in the circulating number of eosinophiles was induced 60 minutes after insulin injection which was not related to any change in the adrenal

TABLE 1. CHANGE IN THE NUMBER OF EOSINOPHILES/CU.MM. OF BLOOD AFTER DIFFERENT EXPERIMENTAL PROCEDURES

EXPERIMENTAL CONDITION <sup>1</sup>	TIME AFTER TREATMENT min.	EOSINOPHILES/CU.MM. OF BLOOD			
		Mean		Dif. $\pm$ S.E.	P of Diff. <sup>2</sup>
		Before	After		
Normal Intact					
a) <i>Ad libitum</i> fed (83).....	3	322 $\pm$ 22			
b) 16-hr. fast (26).....	3	174 $\pm$ 26			0.0001 (a, b)
c) 48-hr. fast (9).....	3	351 $\pm$ 61			0.7 (a, c)
Epinephrine, 0.02 mg/100-gm. rat, s.c.					
d) <i>Ad libitum</i> fed (6) <sup>4</sup> .....	180	602	85	-517 $\pm$ 74	0.0009
e) 16-hr. fast (8) <sup>5</sup> .....	60	230	109	-110 $\pm$ 69	0.1
f) 16-hr. fast (8) <sup>5</sup> .....	180	228	68	-158 $\pm$ 46	0.0035
g) Adrenalectomized (5) <sup>5</sup> .....	60	470	551	+80 $\pm$ 126	0.55
h) Adrenalectomized (12) <sup>4</sup> .....	180	260	1420	+1178 $\pm$ 248	0.0005
i) Splenectomized (4) <sup>4</sup> .....	180	549	77	-462 $\pm$ 32	0.005
j) Adrenalect.-splenect. (5) <sup>4</sup> .....	180	513	713	+200 $\pm$ 184	0.33
k) Sham adrenalect.-splenect. (4) <sup>4</sup> .....	180	233	78	-205 $\pm$ 55	0.03
Glucose per os, 2 ml. 50% Solution					
l) 16-hr. fast (14).....	30	171	241	+57 $\pm$ 29	0.06
m) 16-hr. fast (12).....	60	142	133	-9 $\pm$ 29	0.8
n) 16-hr. fast (6).....	180	137	52	-88 $\pm$ 19	0.0014
Insulin, 0.5 U (0.6 ml.) s.c.					
o) 16-hr. fast (9).....	30	230	197	-33 $\pm$ 28	0.25
p) 16-hr. fast (7).....	60	288	387	+95 $\pm$ 35	0.035
q) 16-hr. fast (10).....	120	178	142	-36 $\pm$ 25	0.12
r) 16-hr. fast (6).....	180	81	26	-56 $\pm$ 17	0.008
Surgery					
s) Adrenalectomy (31).....	8-9 days	341	423	+82 $\pm$ 66	0.18
t) Splenectomy (6).....	8-9 days	216	644	+427 $\pm$ 155	0.006
u) Adrenalect.-splenect. (5).....	8-9 days	213	513	+299 $\pm$ 10	0.04
v) Sham splenectomy (6).....	8-9 days	126	185	+59 $\pm$ 34	0.15
w) Sham adrenalect.-splenect. (4).....	8-9 days	405	233	-172 $\pm$ 46	0.04
Control-treated					
x) Physiol. sal. (14).....	30	159	217	+53 $\pm$ 29	0.09
y) Dist. water per os (8).....	30	144	136	-8 $\pm$ 45	0.9

<sup>1</sup> The numbers in parentheses are the numbers of rats in the groups. <sup>2</sup> Figures in italic type indicate a significant difference. <sup>3</sup> The rats were killed between 9 A.M. and 12 noon. <sup>4</sup> Three injections of epinephrine at successive hourly intervals. <sup>5</sup> A single injection of epinephrine.

ascorbic acid concentration. Particularly relevant to these results is the report (13) that an eosinopenia of equal extent was induced 3 hours after epinephrine or insulin injection in normal man, but not in Addisonian or hypopituitary patients.

Hyperglycemic levels were induced in 3 groups of rats following the administra-

tion of 2 ml. of a 50 per cent glucose solution (table 2). The results show that the adrenal ascorbic acid concentration was not changed by this procedure in any of the groups. However, a significant decrease in the number of eosinophiles per cu. mm. of blood had been induced in the group 180 minutes after the glucose administration. Steeples and Jensen (10) reported that oral glucose administration "leads to an inhibition of the release of the hormones of the adrenal cortex as indicated by an

TABLE 2. RELATION OF THE ADRENAL ASCORBIC ACID CONCENTRATION AND CHANGE IN THE NUMBER OF EOSINOPIHILES/CU.MM. OF BLOOD AFTER PROCEDURES INDUCING HYPOGLYCEMIC OR HYPERGLYCEMIC CONDITIONS

EXPERIMENTAL CONDITION <sup>1</sup>	TIME AFTER TREAT- MENT	ADRENAL ASCORBIC ACID CONCENTRATION		BLOOD GLUCOSE Mean $\pm$ S.E.	SIGNIFICANT CHANGE (+/-)	
		Mean $\pm$ S.E.	P of Diff. <sup>2</sup>		Ascorbic acid conc.	Eosino- philes (from table 1)
	min.	mg. %		mg. %		
Normal Intact						
a) <i>Ad libitum</i> fed (25) <sup>3</sup> .....		365 $\pm$ 16.1		225 $\pm$ 18.9	+	-
b) 16-hr. fast (11) <sup>3</sup> .....		420 $\pm$ 21.3	0.05 (a, b)	157 $\pm$ 5.9		
c) 48-hr. fast (8) <sup>3</sup> .....		435 $\pm$ 35.8	0.08 (a, c)	87 $\pm$ 2.8		
Control-Treated						
d) Physiol. sal (14).....	30	359 $\pm$ 14.8	0.04 (b, d)	180 $\pm$ 6.2	-	
e) Water <i>per os</i> (8).....	30	367 $\pm$ 34.6	0.22 (b, e)	192 $\pm$ 8.2		
Insulin, 0.5 U (0.6 ml.) s.c.						
f) (10) <sup>4</sup> .....	30	341 $\pm$ 15.9	0.45 (d, f)	117 $\pm$ 13.0	-	+
g) (7).....	60	317 $\pm$ 21.4	0.12 (d, g)	105 $\pm$ 7.5		
h) (10).....	120	263 $\pm$ 14.0	0.001 (d, h)	102 $\pm$ 5.1		
i) (6).....	180	266 $\pm$ 5.1	0.001 (d, i)	97 $\pm$ 5.8		
Glucose <i>per os</i> , 2 ml. 50% solution						
j) (9).....	30	316 $\pm$ 20.3	0.25 (e, j)	228 $\pm$ 12.0		-
k) (12).....	60	317 $\pm$ 13.2	0.15 (e, k)	242 $\pm$ 18.5		
l) (6).....	180	425 $\pm$ 23.2	0.15 (e, l)	169 $\pm$ 5.3		
Epinephrine, 0.02 mg/100-gm. rat, s.c.						
m) (6).....	60	274 $\pm$ 20.2	0.003 (d, m)	270 $\pm$ 11.8	-	-
n) (8).....	180	405 $\pm$ 13.5	0.03 (d, n)	239 $\pm$ 14.1	+	
o) <i>Ad libitum</i> fed (8)..... (3 inj. at hourly inter- vals)	180	279 $\pm$ 10.4	0.001 (a, o)	162 $\pm$ 17.5	-	

<sup>1</sup> The numbers in parentheses are the numbers of rats in the groups. <sup>2</sup> Figures in italic type indicate a significant difference. <sup>3</sup> The rats were killed between 9 A.M. and 12 noon. <sup>4</sup> All rats in this and the following groups were overnight-fasted (16 hr.) unless otherwise noted.

increase in the adrenal cholesterol content." The data of Steeples and Jensen and our data agree that the adrenal glands apparently were not stimulated following glucose *per os* insofar as cholesterol or ascorbic acid concentration at a given moment is indication of its activity. Since an eosinopenia was demonstrated in our group of rats 180 minutes after glucose administration, one must consider, on the basis of evidence in the literature regarding this change in the eosinophile picture, that adrenal cortical activity had been affected by this procedure (14).

The relation of adrenal ascorbic acid concentration with changes in the number of circulating eosinophiles after the administration of epinephrine is shown in table 2. Inspection of these data show that a depression in the number of eosinophiles per cu. mm. of blood does not necessarily have to be associated with a fall in the adrenal ascorbic acid concentration even following epinephrine injection. It is apparent from these data, however, that if the epinephrine dose is high enough that the depression of the adrenal ascorbic acid concentration was associated with a significant fall in the number of circulating eosinophiles.

It is noteworthy that the blood glucose levels in the groups of rats after epinephrine and glucose *per os* were comparable, yet the adrenal ascorbic acid concentration was not influenced in any of the latter groups but was significantly depleted in 2 of the groups injected with epinephrine. However, on the basis of the change in the number of eosinophiles per cu. mm. of blood both epinephrine and glucose *per os* had been equally effective 180 minutes after their administration and would have been used to indicate equal stimulation of adrenocortical activity (15). Two other instances of significant depression of the number of circulating eosinophiles not associated with depletion of the adrenal ascorbic acid concentration are presented in the data of table 2. The group of normal intact rats after an overnight fast and the group of rats 180 minutes after a single small dose of epinephrine presented evidence of significant increase of adrenal ascorbic acid concentration associated with a significant fall in the number of circulating eosinophiles per cu.mm. of blood. An explanation of this relationship is suggested in the DISCUSSION.

#### DISCUSSION

Evidence has been presented that the number of eosinophiles per cu.mm. of peripheral blood can be altered in either direction by several different experimental procedures. What happens to the eosinophiles when they disappear from the peripheral blood was not investigated; but it is evident from the data presented in table 1 that the spleen was not the site of mobilization since an eosinopenia was induced in splenectomized rats after adrenaline injection. A significant increase in the number of circulating eosinophiles was induced in groups of rats after splenectomy, adrenalectomy-splenectomy and after epinephrine injection in adrenalectomized rats. This was not associated with any change in the ascorbic acid concentration of the adrenal glands or the spleen (3). A similar pattern of eosinophile change and no change in adrenal ascorbic acid concentration was induced 60 minutes after an injection of insulin. The nature of the mechanism responsible for inducing a significant increase in the number of circulating eosinophiles is not indicated in the present data. The data, however, do indicate that the spleen may have a role in the eosinophilic response.

A significant decrease in the number of eosinophiles per cu.mm. of blood was induced in some of the groups of rats consequent to the administration of insulin, epinephrine, glucose *per os* and after an overnight fast in normal intact rats. It is suggested that the comparable results of eosinopenia associated with significant depletion of the adrenal ascorbic acid 180 minutes after epinephrine or insulin injection can be explained if one assumes that the hypoglycemia which followed the latter injection liberated endogenous epinephrine. The apparent discrepancy of an

eosinopenia associated with a significant increase of adrenal ascorbic acid in the groups of rats after an overnight fast and 180 minutes after glucose *per os* is probably an indication of increased adrenocortical function as a consequence of mildly induced stress. Sayers *et al.* (16) reported that the adrenal ascorbic acid concentration increased in rats approximately 15 hours after a single injection of ACTH.

It is evident from the data that certain agents which induce hypoglycemic or hyperglycemic blood levels can induce an eosinopenia comparable to that which follows the administration of epinephrine. However, this change in the eosinophilic leucocyte blood picture is not always associated with a significant depletion of the adrenal ascorbic acid at the time. This does not necessarily indicate that adrenocortical activity has not been mildly increased, since the rate of adrenocortical hormonal secretion and replenishment has not been established nor the direct association of ascorbic acid concentration with this process. It appears probable that the hypoglycemic and hyperglycemic alteration of the blood level does not *per se* induce the eosinopenic response via an influence on adrenocortical activity. The role of the adrenal medulla in mediating the effect of altered blood glucose levels is under investigation.

#### SUMMARY

The change in the number of circulating eosinophiles was determined and tested for statistical significance in groups of rats subjected to the following procedures: ablation of the adrenal glands, spleen or both organs; after the injection of epinephrine in adrenalectomized, splenectomized or adrenalectomized-splenectomized rats; after an overnight or 48-hour fast of normal intact rats; and after the administration of epinephrine, insulin or glucose *per os* in normal intact rats.

A significant increase in the number of eosinophiles per cu.mm. of blood was induced in those untreated groups in which the spleen was absent; after epinephrine in adrenalectomized rats; and 60 minutes after insulin injection in intact rats. A significant decrease in the number of eosinophiles per cu.mm. of blood was induced only in those groups of rats in which the adrenal glands were *in situ* 180 minutes after epinephrine, insulin or glucose *per os* but not after shorter intervals; and after an overnight fast. The relation of these results to the possible effects of hypo- and hyperglycemia on adrenocortical activity is discussed.

A relationship of induced eosinopenia with significant depletion of the adrenal ascorbic acid was demonstrated only in the 2 groups of rats 180 minutes after the injection of epinephrine or insulin. Significant changes in the eosinophilic leucocyte picture were not associated with significant change in the adrenal ascorbic acid concentration, and the converse also occurred in several groups of rats.

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# SPONTANEOUS ACTIVITY IN RATS FED AN AMINO ACID-DEFICIENT DIET

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ONE of the simplest manifestations of physiological change at the behavioral level is seen in variations in level of spontaneous activity concomitant with some change in diet or endocrine balance or the administration of some drug. While the greater number of experiments concerned with the reflection of internal change in activity level have dealt with drugs, hormones, or vitamins, the recently claimed effects of certain amino acids on learning and intelligence (1-3) make imperative a more thorough study of the relationship between diet composition and spontaneous activity level if a meaningful interpretation of these last mentioned influences is to be attained.

A number of studies report changes in activity level with reduced food intake as well as with deprivation of specific dietary constituents. A summary of this work may be obtained from Reed's recent review (4).

Only one article to date has been concerned with the influence of specific amino acid deficiencies upon psychological functions. Reiss and Block (3) report that while rats fed lysine- and cystine-deficient diets showed statistically significant impairment in maze learning, when compared with their controls, the experimental and control groups showed no reliable differences in general activity or in the strength of the experimentally induced dominant drive operating in the maze-learning situation. Accordingly, they conclude that both lysine and cystine deficiencies produce some central nervous system impairment which affects the animals' maze-learning ability. These experimenters, however, made only a limited number of observations on small numbers of animal subjects. Their animals were placed in Spaeth wheels for extremely short periods of time and apparently allowed to commute *ad libitum* between the wheel and feeding compartment. The animals, furthermore, presumably were not allowed the preliminary adaptation to the wheels that is generally regarded to be highly important. The present writers are therefore not convinced that Reiss and Block have established the Null hypothesis with regards to their activity wheel data. Should differences be shown to exist, poor maze learning on the part of the experimental animals might be accounted for more parsimoniously in terms of decrement in performance rather than in ability. Hence it is the primary aim of the present investigation to provide less controversial data on level of spontaneous activity in amino acid-deficient animals.

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## PROCEDURE

The subjects in this experiment were 10 male rats of an inbred Wisconsin strain, 47 to 60 days old when experimentation began. They were drawn from four litters split so that an equal number of animals from each litter were assigned to the control and experimental groups.

Each animal was placed in a revolving-drum type of activity cage, with a drum diameter of 12 inches, calibrated according to the method described by Lacey (5). It was confined to the drum for 21 hours out of each 24, the remaining three, 4 to 7 p.m., being spent in the feeding compartment. All 10 subjects were fed finely powdered Purina laboratory chow for a 10-day period of adaptation. On the eleventh day, half (the experimental group) were placed on a diet of the following composition: rolled oats, 22 per cent; cornstarch, 53 per cent; corn oil, 11 per cent; yeast extract, 6 per cent; cod-liver oil, 4 per cent; salt mixture, 4 per cent. The remaining half was continued on Purina chow. The diets of both were kept refrigerated until about one hour before feeding to prevent their becoming rancid.

Roller oats comprised the sole source of protein for the experimental group with the possible exception of a small amount in the yeast extract. The proteins of cereal grains are notoriously low in their content of lysine and oat protein is no exception. The amount of lysine in oats is given as 0.33 per cent in contrast to 2.6 per cent in dry skim milk and 7.2 per cent in blood meal. Textbooks state that lysine must make up one per cent of the rat's diet if this species is to grow and develop normally. It is obvious that the experimental diet was deficient in lysine. It may also have been lacking in certain of the other essential amino acids. It is believed that it contained all the constituents necessary for optimum growth with the exception of the amino acids. The salt mixture contained all the minerals known to be required and the yeast extract and cod-liver oil provided both water-soluble and fat-soluble vitamins. There has been some criticism of the use of yeast extract on the basis of lack of knowledge of its constituents. Since a deficiency arose during its use, it would seem that this objection is beside the point. However, in subsequent experiments it will be replaced by a mixture of purified vitamins, members of the B complex.

## RESULTS AND DISCUSSION

*Weight Changes.* The mean weights of the *E* and *C* groups at the beginning of the experiment were 181.6 and 161.4 gm., respectively (cf. table 1), the former being heavier on the average by 20.2 gm. This difference, however, is not to be considered statistically reliable ( $t = 1.30$ ;  $df = 8$ ;  $P = .30$ ) because of the wide dispersion in each group.<sup>1</sup> The mean weights of the experimental and control groups at the end of the experiment were 174.2 and 295.3 gm., respectively, the former being lighter by 121.1 gm. This difference is highly reliable ( $t = 7.86$ ;  $df = 8$ ;  $P = .001$ ). When intragroup comparisons are made it is found that during the course of experimentation, the *E* group lost an average of 7.4 gm., the *C* group gaining an average of 133.9 grams. The

<sup>1</sup> Intergroup differences were tested for reliability by application of student's  $t$  for small independent samples. Here degrees of freedom = 8. Intragroup differences were tested by  $t$  for related measures; here degrees of freedom = 4. cf. Lindquist, E. F. *Statistical Analysis in Educational Research*. New York: Houghton-Mifflin, 1940, pp. 56-66.

latter difference is statistically significant ( $t = 11.55$ ;  $df = 4$ ;  $P = .001$ ); the former is not ( $t = .40$ ;  $df = 4$ ;  $P = .70$ ). The former gains significance, however, if one considers the fact that the weight of the animals fed the amino acid-deficient diet shows no appreciable change with an increase in age of 125 days. In the same period of time, the control animals, even though allowed only a limited feeding period each day, almost doubled their starting weight. That the animals fed the deficient diet were physically stunted is evident; whether this stunting is due directly to deprivation of particular dietary constituents or to the resultant reduced intake noted in the experimental animals is not clear from the present data.

### Changes in Hemoglobin Concentration

Examination of table 1 also indicates no overlapping between the experimental and control animals with regard to hemoglobin concentration; the highest concen-

TABLE 1. WEIGHT OF CONTROL AND EXPERIMENTAL ANIMALS AT BEGINNING AND END OF EXPERIMENTATION. HEMOGLOBIN CONCENTRATIONS IN THE TWO GROUPS AT COMPLETION OF EXPERIMENTATION

GROUP	WT. AT BEGINNING OF EXPERIMENT	WT. AT END OF EXPERIMENT	HEMOGLOBIN CONCENTRATION
	gm.	gm.	gm/100 cc.
Control 1.....	130	270	16.6
2.....	185	296	14.9
3.....	150	300	17.0
4.....	172	312	15.1
5.....	170	296	15.3
Average.....	161.4	295.3	15.6
Experimental 1.....	140	161	13.6
2.....	182	208	12.2
3.....	195	145	11.5
4.....	205	212	12.8
5.....	187	145	13.0
Average.....	181.6	174.2	12.6

tration in the former being 13.6, the lowest in the latter, 14.9. In line with the norm presented by Griffith and Farris (6, p. 410) for adult albinos (100% = 15.6 gm/100 cc. blood), these data indicate anemia in the experimental animals. This agrees with the findings of Harris, Neuberger, and Sanger (7) who report a reduction in both hemoglobin and red-cell count in young rats on an inadequate lysine intake.

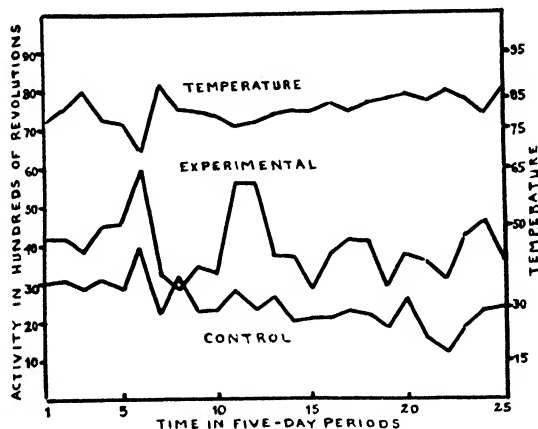
### Activity Changes

Figure 1 represents average daily activity records for the *E* and *C* groups for 25 consecutive 5-day periods. Examination of this graph reveals the activity level of the *E* group to be higher than that of the *C* group for every period except one (period 8). This difference is not statistically reliable for the initial 10 days of adaptation (periods 1, 2) nor the 5 other periods (6, 7, 15, 19, 25). Student's *t* scores gotten for the between-group differences on each of these periods were 1.83 or less ( $df = 8$ ;  $P = .20$  or

greater). It approaches significance on 3 periods (10, 13, 20). For these the  $t$  scores were 1.91, 1.92, 2.15 ( $df = 8$ ;  $P = .10$ ). For all other periods the intergroup difference is regarded to be significant ( $t = 2.31$  or greater;  $df = 8$ ;  $P = .05$  or less). Accepting the criterion for significance provided by the  $t$  scores as valid, the writers regard the groups to have shown no real difference in activity until placed on different diets and attribute the subsequent differences in level to the differential feeding. This is in agreement with previous reports (8, 9) on the initial effects of deprivation but not those on prolonged deficiency. Hitchcock (8), for example, reports the acceleration noted in protein-starved rats passed over into decrement after the third month. The failure of the present  $E$  group to show any clear-cut decline after almost 4 months may be ascribed to the mildness of their deficiency.

Rises and falls in the experimental curve appear as 5 cycles of gradually decreasing length and amplitude. Such shifts are less marked in the control curve. This latter instead shows a general decline terminating with period 22. This appears to be fol-

FIG. 1. MEAN DAILY ACTIVITY LEVELS of experimental and control animals presented for successive 5-day periods. Each point represents mean of 25 observations, 5 observations in 5 animals.



lowed by the beginning of a cycle similar to those seen in the experimental curve. The descending trend is corroborated by the fact that the average activity level for periods 1 to 5 is reliably higher than the levels for periods 11 to 15 (difference = 653 revolutions;  $t = 4.04$ ;  $df = 4$ ;  $P = .01$ ) and 21 to 25 (difference = 1140 revolutions;  $t = 5.04$ ;  $df = 4$ ;  $P = .01$ ). That the difference between the averages for 11 to 15 and 21 to 25 (487 revolutions) is only suggestive ( $t = 2.31$ ;  $df = 4$ ;  $P = .10$ ) can be accounted for by the reversal in trends beginning with the 22nd period.

The presence of cycles in the experimental curve agrees with the findings of Wald and Jackson (10). It may be similarly interpreted. The nutritionally deficient animal displays a lowered threshold of irritability reflected in heightened running activity. This increased activity leads in turn to more rapid physiological depletion. As the animal nears exhaustion, he becomes more quiescent. This continues until some 'repair' takes place with the resultant increased activity. Since diet is inadequate, 'repair' is incomplete; *post hoc* the trend toward quiescence sets in progressively earlier in each cycle. Therefore each cycle gradually decreases in length and amplitude.

The shape of the control curve is also amenable to interpretation in terms of the depletion hypothesis. Only one significant cycle, *periods 5 to 7*, occurs in this curve prior to *period 22*. This is probably due to inadequate temperature control, especially marked during these periods. If this be the case, then it appears as if mild states of deficiency, such as might result from subnormal intake produce lethargy. This is seen in the gradually reduced running. When the deficiency reaches a critical level, irritability increases to an extent that results in increased running, the stage seen in the *E* group. The *C* group never reached the 22.9-gm. daily average intake of food reported by Siegel (11) for 168- to 188-day-old animals on 24-hour free feeding.

### Changes in Food Intake

Although the food intake for the *E* and *C* groups does not differ significantly during the 10-day adaptation period, a reliable difference appears within the first 5 days of regulated feeding. For *period 3* the mean difference was 5 gm. ( $t = 8.90$ ;  $df$

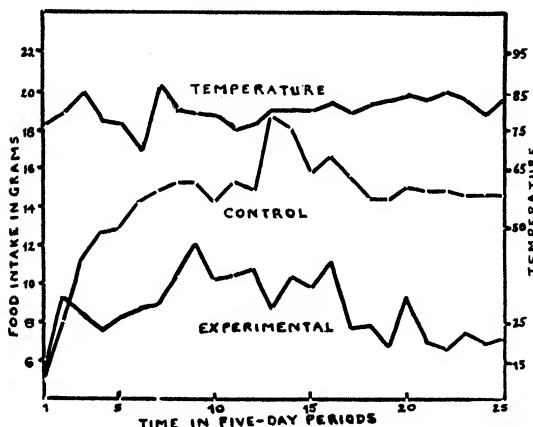


FIG. 2. MEAN DAILY FOOD INTAKE of experimental and control animals presented for successive 5-day periods. Each points represents mean of 25 observations, 5 observations in 5 animals.

= 8;  $P = .001$ ). By the end of the experiment, the mean consumption of the *E* group was reduced to approximately half that of the *C* group, a relationship persisting after *period 13*. For only one period, 9, is the intergroup difference (3.2 gm.) not clearly significant ( $t = 2.13$ ;  $df = 8$ ;  $P = .10$ ). All other  $t$  scores are 2.90 or larger ( $df = 8$ ;  $P = .02$  or less). Ingestion of an amino acid-deficient diet by rats results then in reduced food consumption just as does the ingestion of a diet low in the B complex vitamins (12). Figure 2 clearly reveals these intake differences. The control curve is negatively accelerated, its rapid rise in the early periods and its deacceleration typical of growth curves. Leveling off appears at *period 8*, when the animals were approximately 130 days old and hence had reached young adulthood. The experimental curve, in contrast, shows a smaller increase persisting up to *period 9*. This increase, as in the controls, is attributed to growth. The smaller increment is probably due to the malignant effect of the induced inanition. After *period 9* a decline sets in which is interrupted only by a significant rise between *periods 19 and 21*. The mean difference between 19 and 20 equals 2.5 gm. ( $t = 3.33$ ;  $df = 4$ ;  $P = .02$ ); the mean

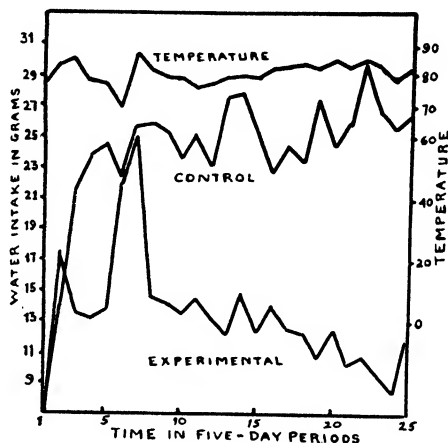
difference between 20 and 21 equals 2.3 gm. ( $t = 4.42$ ;  $df = 4$ ;  $P = .01$ ). This is believed to constitute a brief attempt on the part of the animals in this group to compensate for their depletion.

There are several indications that this decreased intake in the *E* group is associated with the palatability of the deficient diet. Animals allowed to ingest limited quantities of the experimental diet *ad libitum* had food left in their feeding dishes several hours after their controls had completely consumed a similar portion of the powdered Purina chow. Furthermore, rats run through a maze to an experimental diet incentive did not enter the goal box or approach the feeding dish with the vigor or directness displayed by animals run to a Purina chow incentive.

### Changes in Water Intake

The two groups do not differ significantly in water intake during the 10-day adaptation period. However, for every period of differential dietary treatment except

FIG. 3. MEAN DAILY WATER INTAKE of experimental and control animals presented for successive 5-day periods. Each point represents mean of 25 observations, 5 observations in 5 animals.



6 and 7, the intake of the experimental animals is less reliable than that of the controls. The significant intragroup difference for 6 and 7 is due to an unaccountable rise in the consumption of the *E* group during these periods. At the end of the experiment, the intake of the *E* group was less than half of that of the *C* group. Inspection of the control curve in figure 3 indicates a gradual increase in intake which is regarded, as was the increment in food intake, to be a function of the control animals' growth. At the end of the study, the average 27.4 gm. compares favorably with a previously published norm (11). On the other hand, the experimental curve, with the exception of periods 6 and 7, displays a decline after the adaptation period. This is in line with the trend noted in the food-intake data.

### SUMMARY

Ten rats of an inbred Wisconsin strain, 47 to 60 days of age at onset of experimentation were placed in activity wheels for a continuous period of 125 days. Five

were fed Purina laboratory chow during a 3-hour feeding period each day, while the remaining 5 were maintained on an experimental diet deficient in lysine and probably other amino acids. The following results were obtained:

The experimental animals showed no significant weight changes over the entire period of the investigation, as contrasted to the apparently normal weight gain of the control group. The experimental group showed lower than normal hemoglobin concentrations. The activity levels for the experimental group were in general higher than those of the control group, cycles similar to those reported by previous investigators being present in the records of the former.

The food intake of the experimental animals was reliably less than that of the controls and showed a gradual decline. The water intake of the experimental animals was significantly less than that of the controls except during periods 6 and 7, gradually declining during the period of experimental feeding.

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# INFLUENCE OF O<sub>2</sub> CONTENT OF INSPIRED AIR ON TOTAL LUNG VOLUME

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IN 1932 and 1936 it was shown with body plethysmography on man and on rabbits (1-6) that if voluntary muscle work is done, or air with decreased O<sub>2</sub> content is inspired, then besides an increase of the tidal air and rate of respiration, i.e. the total ventilation, an increase of 'total lung volume' also occurs. This increase must lead to an increase of the respiratory surface of the lung. Thoracometry also showed that during muscular work and lack of O<sub>2</sub>, the tonus of the inspiratory muscles is so increased that the thorax rests in an inspiratory position (7, 8). Our plethysmographic work with decreased oxygen content of air was confirmed independently by Harris (9). He saw in cats a great increase of lung volume with 8 per cent O<sub>2</sub> in the inspired air, and found that after vagotomy this reaction disappeared. In our opinion, the tonus of the plain muscles of the bronchioli and the lung is correlated with the increase of tonus of inspiratory skeletal muscle. The former must decrease if the latter increases (3, 4).

Our present problem was to see whether the increase of the 'total lung volume,' on which the tidal air is superimposed, is correlated with the increase of the volume or with the rate of respiration; whether it is connected with every dyspnoic respiration, and especially the way in which the decrease of oxygen and the increase of CO<sub>2</sub> in the respiratory air act (10).

## METHODS AND RESULTS

Former experiments of this laboratory were made with a body plethysmograph with volumetric registration. It seemed necessary to prove whether or not a compensation-plethysmograph such as that used by Bucher, which is uninfluenced by possible temperature differences during an experiment (11), will also show this reaction. As the experiments described herein prove, this is fully the case.

The experiments were made on rabbits and cats, narcotized with 1.0 to 1.2 gm/kg. of urethane s.c. Narcosis with chloralose or with narconumal in cats influences respiration and is not so suitable for such experiments. Cats do not show as regular breathing as do rabbits. The animal was tracheotomized and put in the body-plethysmograph with temperature compensation. The plethysmograph was submerged in a water bath at 20° C. and connected with the compensation room through a pressure capsule. The volume changes, transferred into pressure, were registered

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on an optical kymograph. Blood pressure was registered from the carotid artery with a Broemser optical manometer. The jugular vein was connected with a syringe and Liquemin (Roche) was injected from time to time to prevent blood clotting.

The animal respired through a T-shaped tracheal cannula. Normal air, air diluted with N, or air with the addition of  $\text{CO}_2$ , was supplied from spirometers along the cannula. Figure 1 shows schematically these experimental conditions.

Experiments were generally performed in the following way. First normal respiration was registered for about one minute. Then the air mixture, the action of which was to be studied, was given for 1 to 3 minutes. After that, normal air was given again. Only after 10 to 15 minutes or more was an experiment repeated. Air diluted with N was usually given first and after that, air with  $\text{CO}_2$  was added.

Forty-six experiments were made on 7 rabbits and 18 experiments on 3 cats, with oxygen concentrations between 5 and 8 per cent and  $\text{CO}_2$  concentrations be-

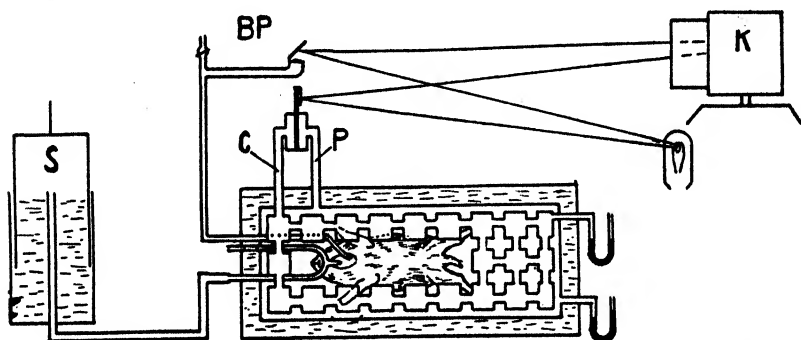


FIG. 1. P, plethysmograph; C, compensation room, S, spirometer, BP, blood pressure; K kymograph.

tween 2 and 10 per cent. Figures 2 and 3 show typical experiments with  $\text{CO}_2$  increase and lack of  $\text{O}_2$  on a rabbit and a cat.

Experiments with decreased  $\text{O}_2$  showed that the inspiratory movements increased very quickly and that the expiratory movements did not return to the base line. That is, the total volume of the body (i.e., of the lung) had increased. When  $\text{CO}_2$  was respired, the ventilation increased, especially the depth of the respiration, but the total lung volume did not increase.

To describe the changes in the rate and the volume of respiration and the minute-volume, if less  $\text{O}_2$  or more  $\text{CO}_2$  was respired, we took the last half-minute of the period in which the modified air was given and compared it with the previous values on normal air. The differences are so expressed that normal values are taken as 1 and modified values as a multiple of this. Thus a 'coefficient of respiration rate' ( $K_r$ ) of 1.5 means that the number of respirations per minute increased from 10 to 15, or from 20 to 30. The same calculation was made for the changes in volume of respiration, the tidal air ( $K_t$ ) and of the respiratory minute-volume, i.e. ventilation ( $K_v$ ).

In figure 4 the coefficient of the rate of respiration  $K_r$ , and the coefficient of

respiratory volume (tidal air),  $K_t$ , are related to each other in all experiments with different quantities of  $O_2$  and  $CO_2$ . The figure shows that oxygen lack increases the rate of respiration,  $K_r$ , relatively more than the volume of respiration,  $K_t$ . Increase of  $CO_2$ , on the contrary, results especially in an increase in the volume of respiration. Exceptionally, however, mostly in rabbits, together with an increase in the volume

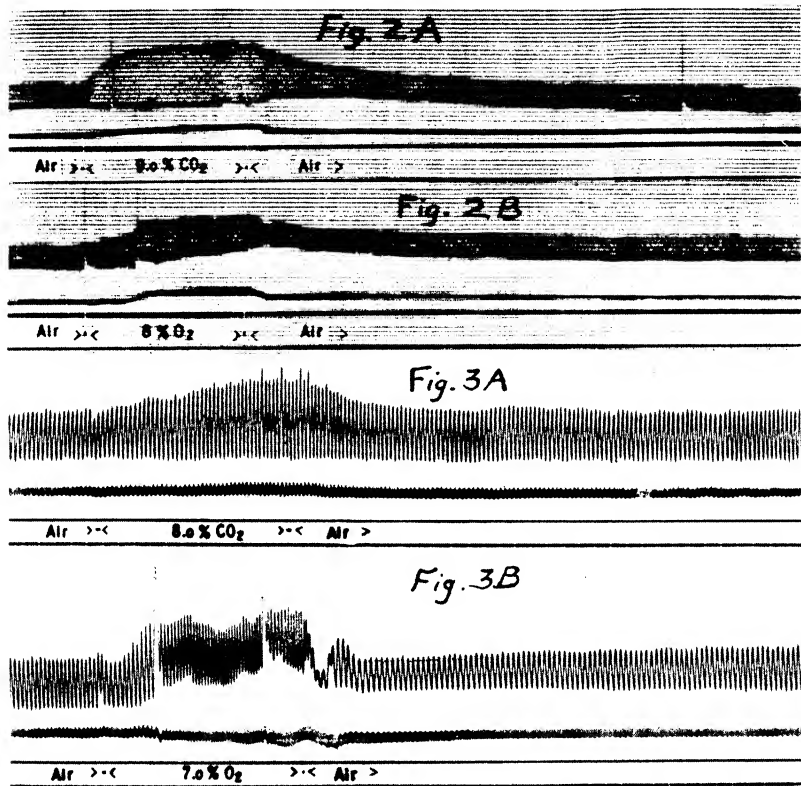


FIG. 2 (upper). RABBIT. *A*, action of 8 per cent  $CO_2$  on body volume and respiration. *B*, action of 8 per cent  $O_2$  on body volume and respiration. Under respiration curve blood pressure curve and time registration are shown.

FIG. 3 (lower). CAT. *A*, action of 8 per cent  $CO_2$  on body volume and respiration. *B*, action of 7 per cent  $O_2$  on body volume and respiration. Under respiration curve blood pressure curve and time registration are shown.

of respiration there was also observed an increase of the respiratory rate. The straight line crossing figure 4 gives an approximate division between experiments in which each of these relations mostly appeared. Above the line are all experiments with lack of oxygen in cats and most of the experiments in rabbits, with only 7 exceptions for the latter. Under the line are all experiments with increase of  $CO_2$ , with only 4 exceptions in rabbits and one in a cat.

The main object of this study was to see how the *total lung volume* changes under these circumstances. We compared the increase of lung volume with changes in respiration rate,  $K_r$ , (fig. 5) respiratory volume,  $K_t$  (fig. 6), and minute ventilation,  $K_v$

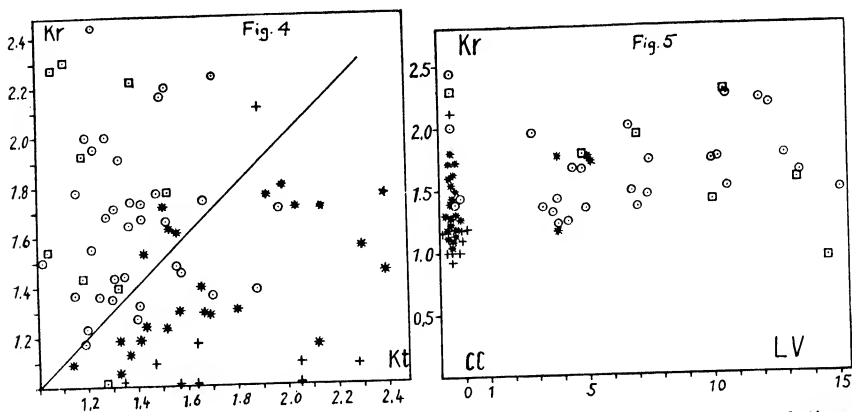


FIG. 4 (left). Abscissa: Increase of tidal air (respiration volume),  $K_t$ ; normal = 1. Ordinate: Increase of rate of respiration,  $K_r$ ; normal = 1.  $O_2$  experiment: circles stand for rabbit. Squares stand for cat.  $CO_2$  experiment: Asterisks stand for rabbit. Plus signs stand for cat.

FIG. 5 (right) Abscissa: Increase of lung volume (LV) in cc. Ordinate: Increase of rate of respiration ( $K_r$ ); normal = 1.

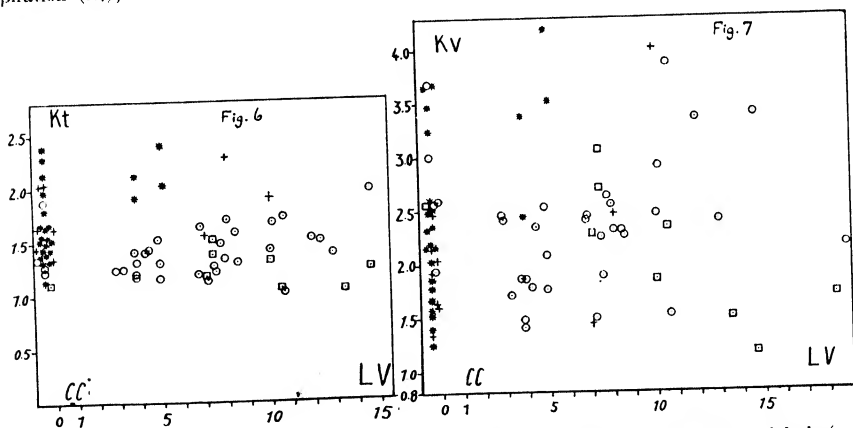


FIG. 6 (left). Abscissa: Increase of lung volume (LV) in cc. Ordinate: increase of tidal air (respiration volume),  $K_t$ ; normal = 1.

FIG. 7 (right) Abscissa: Increase of lung volume (LV) in cc. Ordinate: increase of minute volume ( $K_v$ ), ventilation/min.; normal = 1.

(fig. 7). In figure 5, lung-volume increase is related to the change of respiratory rate. It was found that, with a few exceptions, only those experiments with  $O_2$  lack showed an increase in lung volume, while those with added  $CO_2$  did not. In those experiments in which  $CO_2$  increased the rate of respiration as much as the lack of

O<sub>2</sub>, the lung volume still did not increase. Thus the increase of lung volume bears no relation to the increase in the rate of respiration. The increase of lung volume after lack of O<sub>2</sub> was between 3 and 15 cc.

It was further found that there was no relation between increase of respiratory volume (tidal air) and total lung-volume increase. In experiments with CO<sub>2</sub> the respiratory volume was always increased. With lack of oxygen there was generally also a small increase, which however seldom was more than  $K_l = 1.7$ . With CO<sub>2</sub> there was generally no increase in the lung volume, while in nearly all cases with lack of oxygen the lung volume increased considerably. Finally, as shown in figure 7, the respiratory minute volume,  $K_r$ , was related to the increase of lung volume. All experi-

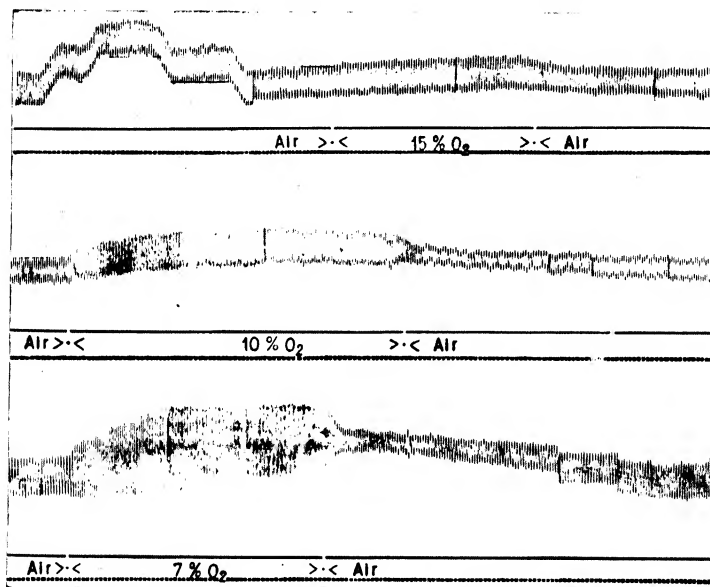


FIG. 8. ACTION of different O<sub>2</sub> concentrations in air on body volume and respiration on a rabbit. A. Calibration: each step = 10 cc.; B. action of 15 per cent O<sub>2</sub>; C. action of 10 per cent O<sub>2</sub>; D. action of 7 per cent O<sub>2</sub>. *Upper curve*: body volume (respiration), *middle curve*: signal, *lower curve*: time in 10 sec.

ments (O<sub>2</sub> and CO<sub>2</sub>) showed an increase of ventilation with  $K_l = 1.2$  to 4.2. There was no obvious correlation between the increase of ventilation and the increase of lung volume. In most CO<sub>2</sub> experiments, in spite of ventilation increase, there was no lung-volume increase. In experiments with oxygen lack, in nearly all cases the total lung volume increased; but again it would be impossible to say that there was any certain correlation with the increase of ventilation.

Thus it seems to be clear that increase of total lung volume is not related to the increase in the rate of respiration, the respiratory volume, nor to the minute volume of respiration. It appeared in by far the greatest number of experiments with lack of oxygen and was not present with increase of CO<sub>2</sub>. There are a number of exceptions,

but even in these a comparison of the quantitative results shows the difference between  $O_2$  and  $CO_2$  experiments. Thus, in four  $CO_2$  experiments the respiratory volume increased ( $K_l = 1.9$  to  $2.4$ ) and the lung volume increased 3 to 5 cc. With lack of oxygen, however, if the respiratory volume increase,  $K_l$ , was only 1.1 to 1.7, the lung volume increased much more, mostly between 5 and 15 cc. It is concluded from these experiments that lack of oxygen was the stimulus for the increase of lung volume.

The question then arises whether or not the lung volume increases parallel with the decrease of oxygen in the inspired air. Thirty experiments on 4 rabbits were performed with normal air compared with air-nitrogen mixtures of 15, 12, 5, 10 and 7 per cent oxygen. The strength of the reaction varied considerably. The lung-volume

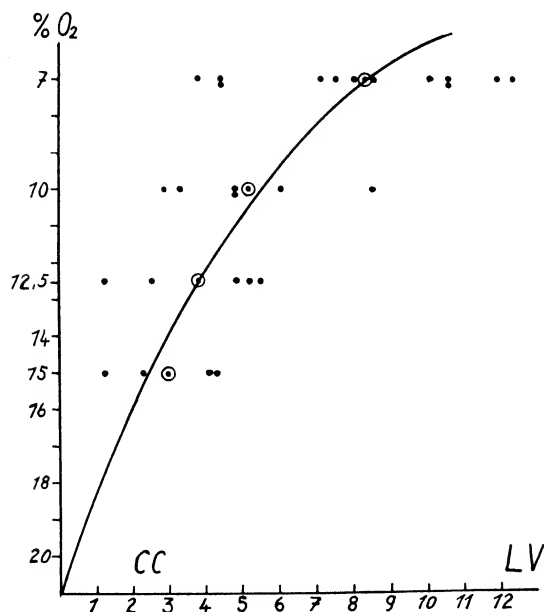


FIG. 9. *Abscissa:* Increase of lung volume (LV) in cc. *Ordinate:*  $O_2$  per cent in air. *Circles,* mean values; *dots,* single experiments.

reaction increased with the decrease of the oxygen content in the inspired air. Figure 8 gives an example of such an experiment with different oxygen mixtures of 15, 10 and 7 per cent  $O_2$ . On the left top side a calibration of the plethysmograph with 10 cc. of air is shown. Figure 9 shows the increase of the lung volume related to the oxygen content of the inspired air. The increases are not very regular, but the middle values for each oxygen concentration show clearly the increase of the lung volume with the decrease of the oxygen content along a hypothetical curve.

It would be better, of course, to correlate the lung volume not with the oxygen content of the air but with the oxygen content of the blood, which falls according to the dissociation curve of oxyhemoglobin. Individual differences of the strength of reaction in different animals, or in the same animal at different periods, might be related partly to differences in the excitability of the respiratory center under the influence

of the narcosis, or to previous experiments, which have led to changes in the state of the respiratory center.

#### DISCUSSION

The increase of total lung volume is the result of an expanded inspiratory position of the thorax and diaphragm, resulting from an increased tonus of the inspiratory muscles. This must lead to an increase of the total lung volume and successively to an increase of the respiratory surface of the lung (2, 4). In the present experiments the influence of  $O_2$  lack and  $CO_2$  increase in the respiratory air was studied. Only  $O_2$  lack proved to give this effect regularly, while increase of  $CO_2$  did not. The lung-volume reaction increases with the decrease of oxygen in the inspired air. No correlation was found with the increase of respiration rate, respiration volume and respiratory minute volume. Thus the increase of inspiratory muscular tonus is a result of lack of oxygen in the inspired air.

The immediate increase of total lung volume in certain voluntary muscular work (1) can hardly be the result of lack of oxygen. This might be due to a direct stimulation of the respiratory center. It is supposed that the result of this increase of the total lung volume during oxygen lack and voluntary muscular work, which leads to an increased respiratory surface, is that it facilitates the diffusion of oxygen into the blood.

#### SUMMARY

Measurements of changes of total lung volume during respiration were carried out with a body-plethysmograph on rabbits and cats narcotized with urethane. Lack of oxygen (5-8%  $O_2$ ) regularly increased the lung volume;  $CO_2$  (2-10%) in the inspired air had no such effect, in spite of increased ventilation. Lack of oxygen mainly increased the number of respirations,  $CO_2$ , mainly the respiratory volume. There was no quantitative correlation between the increase of the total lung volume and the increase of the rate, the increase of the volume of respiration (tidal air) and the increase of the respiratory minute volume.

The factor which influences the total lung volume is the lack of oxygen. Lung volume increases as the oxygen content of the inspired air decreases.

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# ROLE OF PULMONARY PROPRIOCEPTIVE REFLEXES IN SUPPRESSION OF SPONTANEOUS BREATHING DURING ELECTROPHRENIC RESPIRATION<sup>1</sup>

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IT HAS recently become possible to maintain a normal respiratory exchange in animals and man by periodic stimulation of one phrenic nerve through an implanted or surface electrode (1-4). During the application of this 'electrophrenic respiration' an inhibition of spontaneous breathing can invariably be achieved in man and almost invariably in anesthetized experimental animals. This inhibition has been previously shown to be mediated by two mechanisms (2): *a*) by hyperventilation and *b*), more immediately, by afferent impulses in the vagi resulting from the descent of the diaphragm and the distention of the pulmonary parenchyma. Since this reflex inhibition is of obvious clinical significance (e.g. in bulbar poliomyelitis) and of considerable theoretical interest, the present investigation was undertaken to examine those components of electrophrenic respiration which most easily produce this phenomenon.

## METHODS

Dogs weighing 9 to 16 kg. were used, anesthetized with sodium pentobarbital (Nembutal, 30 mg/kg.). A tracheal cannula was routinely introduced. Stimuli were applied directly to the *right* phrenic nerve through a pair of shielded silver wire electrodes. The phrenic and vagi were exposed in the neck. The abdomen was opened wide and two insulated copper-wire electrodes, bared for a millimeter about 1 cm. from the tips, were looped securely into the *left* (unstimulated) leaf of the diaphragm. Diaphragmatic action potentials were then led through small condensers into a condenser-coupled amplifier on push-pull and thence to a cathode-ray oscillograph and loudspeaker, thus revealing respiratory activity both during normal breathing and during stimulation of the right phrenic nerve. In a few instances, action potentials from inspiratory intercostal muscles were similarly studied.

The stimulator used delivers biphasic shocks (duration, 4 msec.) at a frequency of 60 per second. The voltage rises and falls rhythmically causing a diaphragmatic contraction which resembles that of a normal respiration. The output of the stimulator has three variables which can be changed separately—the maximum voltage attained in each cycle, the length of each cycle (which determines the rate of respira-

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tion), and finally the per cent of each cycle occupied by inspiration (inspiration ratio).

## RESULTS

*A. Conditions Which Favor Inhibition of Spontaneous Respiration.* 1. *Rate of respiration.* At any given depth and duration of inspiration during electrophrenic respiration, inhibition of spontaneous breathing may not occur if the rate of artificial respiration is too slow. Under these conditions, increasing the rate of electrophrenic respiration can produce inhibition. Figure 1 shows the result of a typical experiment in which increasing the rate of electrophrenic respiration resulted in a suppression of previously uninhibited spontaneous breathing.

2. *Duration of inspiration.* At any given rate and depth, a minimum duration of inspiration (inspiration ratio) during any one respiratory cycle is necessary for

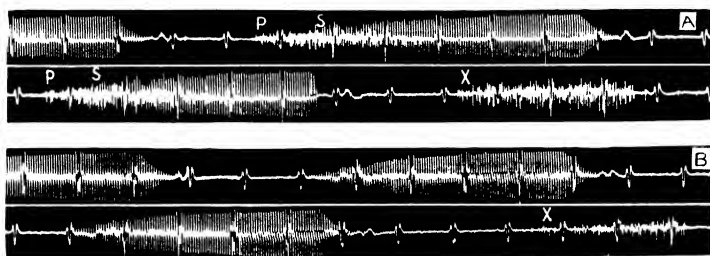


FIG. 1. THIS AND ALL SUBSEQUENT RECORDS are of electrical activity in left leaf of diaphragm during application of electrophrenic stimulation to right phrenic nerve. EKG is also seen in all records as di- or triphasic deflection. Time in this and all subsequent records may be judged from stimulus artefacts, which have a frequency of 60 per second. A: Showing last 3 of short train of electrophrenic stimulations (shown by stimulus artefacts) followed by diaphragmatic action potentials of a normal breath (at X). Spontaneous respiratory rate, 22 per minute. Electrophrenic respiration applied at rate of 18.3 per minute. Spontaneous respiration not inhibited, as shown by occurrence of action potentials (beginning at P) superimposed on beginning of stimulus artefacts (first well seen at S). Continuous record. B: Same preparation, 10 minutes later. Similar record. Spontaneous rate now 18 per minute. Electrophrenic stimulation applied at rate of 20.6 per minute resulted in suppression of spontaneous activity. Only stimulus artefacts are seen until electrophrenic respiration is terminated and spontaneous burst of action potentials comes through (at X), indicating resumption of spontaneous respiration. Continuous record. Sept. 30, 1949.

suppression. For example, figure 2A shows a failure to inhibit spontaneous respiration with an electrophrenic rate of 14 per minute and an inspiration ratio of 61 per cent.<sup>2</sup> Lengthening the duration of inspiration to 83 per cent<sup>2</sup> of the cycle *without changing the rate or depth* abolished spontaneous respiration (fig. 2B) which returned when electrophrenic respiration was terminated (fig. 2C).

3. *Depth of inspiration.* Depth of inspiration during electrophrenic respiration is governed by the maximum voltage applied to the phrenic nerve. If this is not

<sup>2</sup> These figures represent that portion of the respiratory cycle during which the voltage was building up and falling back to zero, i.e. the 'electrical inspiration.' Since a certain threshold had to be reached before the descent of the diaphragm began, actual inspiration occupied a percentage of the cycle that was appreciably less than these figures indicate.



great enough, inhibition of the respiratory center may not occur. Raising the voltage alone (as, for example, from 1 to 1.5 volts), while keeping the rate and inspiration ratio constant, causes a deeper inspiration. Central inhibition will then occur (fig. 3).

*B. Dependence of Inhibition on Vagal Afferent Impulses.* It has previously been shown by another technique (2) that spontaneous respiration is not immediately inhibited during electrophrenic respiration unless the vagi are intact. This finding was confirmed during the present study. Section of either vagus nerve alone abolished the inhibition occurring during electrophrenic respiration (fig. 4).

*C. Effect of Breathing CO<sub>2</sub> on Suppression of Spontaneous Respiration.* Hypercapnia, by increasing the excitability of the respiratory center, might be expected

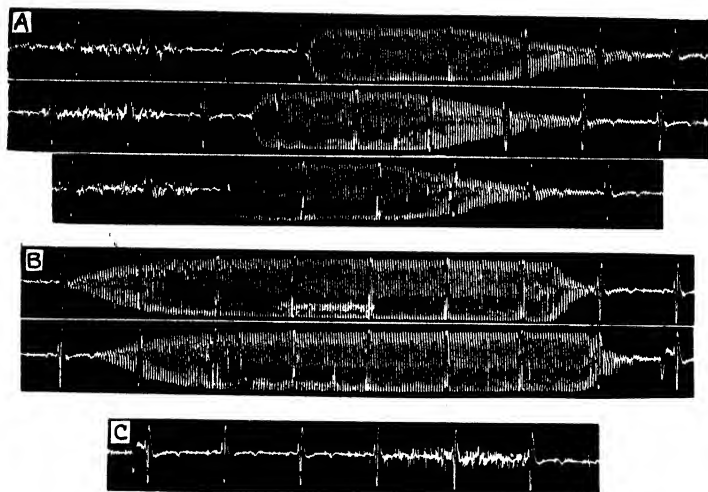


FIG. 2. EFFECT OF INCREASING DURATION OF inspiration (inspiration ratio). *A*: Rate of electrophrenic respiration, 14 per minute. Inspiration ratio, 61 per cent<sup>2</sup>. Spontaneous diaphragmatic activity persists. Continuous record. *B*: Thirty seconds later. Rate and depth of electrophrenic respiration the same. Inspiration ratio increased to 81 per cent<sup>2</sup>. Spontaneous activity now inhibited. Continuous record. Same preparation as *A*. *C*: Same preparation as *A* and *B*. Burst of diaphragmatic action potentials immediately after cessation of electrophrenic respiration, indicating resumption of spontaneous respiration. Record continuous with *B*. Nov. 1, 1949.

to decrease the facility with which spontaneous breathing is suppressed during electrophrenic respiration. This supposition proved to be correct. Figure 5*A* shows a rate, depth, and inspiration ratio capable of inhibiting spontaneous respiration, as shown by the absence of spontaneous action potentials. That this inhibition depends upon the relative state of excitability of the respiratory center is shown in figure 5*B*, in which the conditions of stimulation were precisely the same except that 5 per cent CO<sub>2</sub> and 95 per cent O<sub>2</sub> had been administered to the animal, whereupon spontaneous respiratory activity resumed.

*D. Suppression of Intercostal Activity.* In a few experiments inspiratory intercostal action potentials were studied. Electrophrenic respiration inhibited intercostal motor activity in the same way as that of the diaphragm.

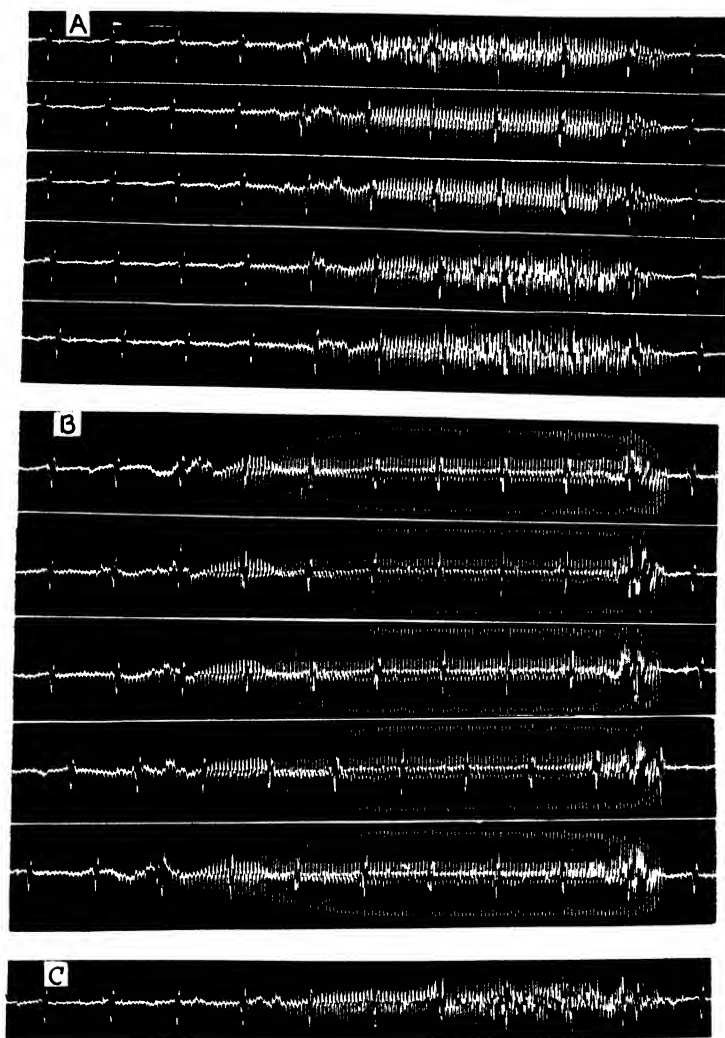


FIG. 3. EFFECT OF DEPTH OF INSPIRATION. *A*: Five consecutive electrophrenic stimulations at 1 volt. Resulting inspirations are not deep enough to inhibit respiratory center, and spontaneous activity is seen coincident with first, fourth, and fifth artificial breaths. *B*: Same preparation, rate, and inspiration ratio, but voltage applied to phrenic has been increased to 1.5 volts, as shown by larger stimulus artefacts. Five consecutive electrophrenic breaths with absence of spontaneous activity. First electrophrenic stimulation delivered about 3 seconds after last breath in *A*. *C*: Same preparation. Next breath after *B*, after return to 1 volt on stimulator. Spontaneous activity again breaking through. Oct. 11, 1949.

#### DISCUSSION

Inhibition of spontaneous breathing during electrophrenic respiration was shown above to be favored by an increase in the rate of artificial respiration, an increase in

the depth of inspiration, and an increase in the duration of inspiration during each cycle. Each of these procedures would be expected to increase the number of afferent impulses ascending the vagi. The impulses active in suppression of spontaneous breathing are presumably those produced by expansion of the lungs and serving to limit the inspiratory effort (5).

It is well known (6) that the inspiratory center has a sharply defined threshold for inhibition, and that the respiratory centers undergo cyclic changes in excitability

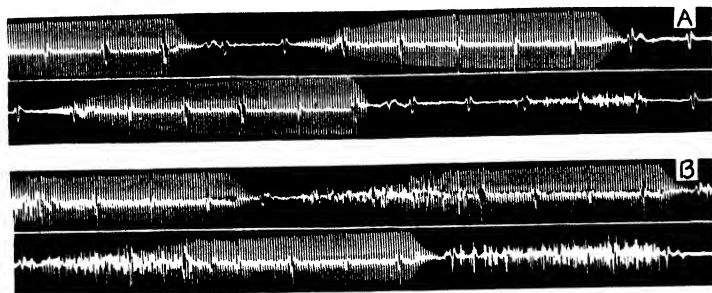


FIG. 4. DEPENDENCE OF INHIBITION ON integrity of vagi. *A*: Continuous record showing inhibition of spontaneous respiration during 3 electrophrenic breaths. At conclusion of artificial respiration, spontaneous burst occurs. *B*: Same preparation, 2 minutes later. Right vagus has been sectioned. Spontaneous diaphragmatic activity occurring superimposed on stimulus artefacts of electrophrenic respirator. Continuous record. Sept. 30, 1949.

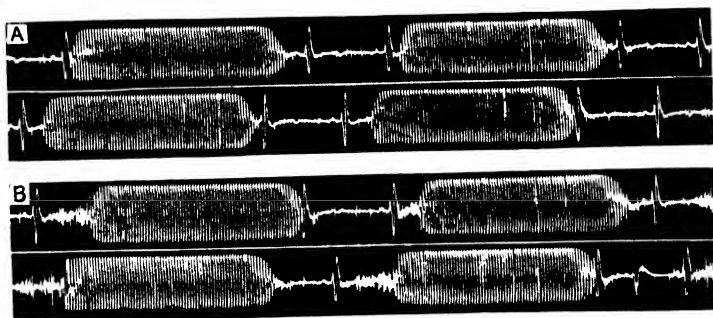


FIG. 5. EFFECT OF HYPERCAPNIA. Continuous records. *A*: Four consecutive electrophrenic breaths with complete inhibition of spontaneous respiration. *B*: Same preparation 9 minutes later, after dog had been breathing 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{O}_2$  for 6 minutes. Spontaneous activity now occurring at beginning of each electrophrenic breath, presumably due to decreased susceptibility of center to inhibition. Nov. 1, 1949.

(7), so that they are more easily inhibited during the latter part of the inspiratory phase. Our data can be explained on the basis of these facts. Increasing the rate of electrophrenic respiration presumably not only increases the total inhibitory bombardment of the center, but also results in a greater chance of delivering the inhibitory impulses to the center at phases of the cycle when it is more susceptible to inhibition. Similarly, increasing the depth and duration of inspiration should bombard the center with more impulses (8) which become effective inhibitors of spontaneous respiration when their number is sufficient to exceed the threshold of the center.

It thus appears probable that the suppression of respiration during electrophrenic stimulation occurs because the inhibitory bombardment of the center by vagal afferent impulses is of sufficient magnitude to reduce its excitability to the extent that it cannot discharge spontaneously. However, the data of *section C* show that even when the respiratory center is successfully suppressed the threshold for inhibition can be raised by increasing the excitability of the center with  $\text{CO}_2$ , so that the number of afferent impulses previously required for inhibition is no longer adequate.

In addition to the easily obtained suppression of spontaneous respiration described above, an apparent excitation of the respiratory center can be produced in human subjects and more rarely in anesthetized dogs. When the voltage output of the electrophrenic stimulator is set at a relatively high value, the resultant rapid and strong contraction of the diaphragm may be followed not by a suppression of spontaneous breathing but by an immediate striking inspiratory motion of the thoracic cage. It is reasonable to suppose that this reflex inspiratory activity is due to the excitation of inspiratory-excitatory vagal afferents brought about by the excessive diaphragmatic descent. These afferents have been described as responding to deep inspirations (5).

Experiments somewhat comparable to those reported in this paper were performed by Brigenti (9). He was apparently the first to observe that stimulation of the peripheral end of a cut phrenic nerve resulted in a striking inhibition of spontaneous respiration, and that this effect was mediated by the vagi. We have repeatedly noticed, as he did, that spontaneous respiration seems to be more easily inhibited when the diaphragm is made to contract by stimulation of a phrenic nerve, than when the lungs are inflated by positive pressure, even though the respective tidal volumes are approximately equal. Thus it is possible that the inhibition herein described may be enhanced by the operation of factors other than those concerned solely with simple distention of the pulmonary parenchyma. This aspect of respiratory physiology deserves further investigation.

*Clinical Implications.* Electrophrenic respiration has been found useful in managing the respiratory failure seen in acute bulbar poliomyelitis (10), since the suppression of the defective spontaneous respiration is on a reflex basis and thus more easily obtained than with a tank respirator. If, during electrophrenic respiration, difficulty is encountered in suppressing spontaneous breathing without overventilation and alkalosis, it would seem most logical from the above data to increase the duration of inspiration (inspiration ratio), since this procedure should increase the chance of reflex central inhibition without, at any selected rate and depth, producing any significant increase in ventilation.

#### SUMMARY

The immediate inhibition of spontaneous breathing during electrophrenic respiration in Nembutalized dogs is favored by *a*) increasing the rate of artificial respiration, *b*) increasing its depth (higher voltage applied to phrenic nerve), and *c*) increasing the duration of inspiration in any given respiratory cycle. The inhibition depends at least in part on afferent impulses in the vagi.

Hypercapnia, which is known to increase the excitability of the respiratory

center, elevates the threshold for reflex central inhibition during electrophrenic respiration. Inspiratory intercostal activity is also inhibited during electrophrenic respiration. It is concluded that the immediate suppression of spontaneous breathing during electrophrenic respiration is due to inhibitory vagal afferent impulses which prevent the spontaneous discharge of the respiratory center.

In addition to the usual suppression of spontaneous respiration, reflex excitation of the inspiratory center can be achieved by producing an excessive diaphragmatic contraction. Producing an active descent of one leaf of the diaphragm by stimulation of a phrenic nerve seems to be a more effective stimulus for inhibition of spontaneous respiration than inflation of the lungs by positive pressure. This would suggest the operation of factors other than those concerned solely with simple distention of the pulmonary parenchyma.

The clinical implications of the results are discussed. It is pointed out that prolonging the duration of inspiration in any one cycle should more effectively inhibit spontaneous respiration while not affecting ventilation to any great extent.

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# ADAPTATION TO HISTOTOXIC ANOXIA

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FOR a long time it has been known that anoxia causes stimulation of the hematopoietic system, resulting in an increased red cell count, increased hemoglobin (1) and a reticulocytosis (2). The purpose of the experiments presented here was to ascertain whether or not histotoxic anoxia produces a response of the blood forming organs comparable to that produced by anoxic anoxia.

In order to determine this, red cell counts, hemoglobin determinations and reticulocyte counts were carried out on 6 rabbits. The experimental animals received daily injections of potassium cyanide to produce histotoxic anoxia. Potassium cyanide acts by blocking the oxidase responsible for converting reduced cytochrome to the oxidized form. As is well known, cytochrome is a pigment which acts as a hydrogen ion transporter, essential to cellular respiration (3).

Since it has been reported that anoxic and anemic anoxia cause changes in the size of the erythrocytes (4, 5), it was deemed worthwhile to measure the diameter of the red cells of animals which had received potassium cyanide.

## METHODS

Six adult rabbits were used. Red cell counts and hemoglobin determinations were made daily for 5 to 11 days during the control periods. Red cell diameter measurements were studied on 3 of the rabbits.

Red cell counts were determined in the standard manner. Hemoglobin determinations were done by the Sahli method. Reticulocytes were stained with brilliant cresyl blue in 0.9 per cent saline, a smear made, and counterstained with Wright's stain; 1000 cells were counted in each determination.

Red cell diameters were measured with an eyepiece micrometer. The cells were measured in their own serum by placing a freshly drawn drop of blood within a ring of vaseline on a microscope slide and covering with a cover slip. One hundred cells for each determination were measured under an oil immersion lens (magnification  $\times 970$ ). Only those cells having a good circular appearance and not touching any other cell were measured.

Potassium cyanide in about one cc. of physiological saline was given subcutaneously, beginning with doses of one mg/kg. of body weight; as a tolerance for the cyanide developed, more of the drug was administered. The highest dose given to any animal was 3.3 mg/kg/day. In 3 of the rabbits sufficient cyanide was given to produce definite dyspnea. In the other 3 animals, doses large enough to cause weakness, as evidenced by drowsiness or inability to stand, were used. In order to prevent the possibility of a transient rise or a redistribution of cells, the drug was given after the daily determinations had been made.

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## RESULTS

The results obtained are shown in table 1. All of the animals showed an increase in the red cell count, and in 4 of the 6 the results were statistically significant. The potassium cyanide produced an average rise of 0.47 million cells/mm.<sup>3</sup> over the control period. The hemoglobin showed an average increase of 0.6 gm/100 cc. This effect was seen only in 5 of the 6 animals, and of these, only 2 showed a statistically significant increase.

When the daily counts of all the rabbits were averaged and the mean corpuscular hemoglobin calculated, no significant change was found. In all of the rabbits, the reticulocyte count increased. The results were significant in all but one animal. The

TABLE 1. EFFECT OF POTASSIUM CYANIDE ON THE HEMATOPOIETIC SYSTEM OF THE RABBIT

ANIMAL NO.	CON-TROL	RANGE	EXPTL.	RANGE	<i>p</i> OF DIFF.	CON-TROL	RANGE	EXPTL.	RANGE	<i>p</i> OF DIFF.
<i>Red Blood Cells, millions/mm.<sup>3</sup></i>						<i>Mean Corpuscular Hemoglobin, µg.</i>				
1	5.12	4.40-6.02	5.46	4.78-6.12	.05	22.1	18.7-27.3	21.7	19.6-23.5	
2	5.35	4.29-6.16	5.50	4.40-6.98	.2	20.8	18.7-24.5	21.8	15.7-27.6	
3	4.58	3.85-5.72	5.29	4.26-7.94	.05	23.5	21.0-27.3	25.0	14.2-26.8	
4	4.98	4.65-5.40	5.09	4.60-5.35	.2	23.0	21.9-24.9	22.8	21.3-25.7	
5	5.05	5.46-6.05	6.51	6.20-7.01	.001	24.3	22.1-26.0	22.0	19.7-24.2	
6	5.11	4.90-5.30	5.69	5.12-6.25	.05	23.5	22.5-24.6	21.5	19.2-23.2	
Av.	5.12	3.85-6.16	5.59	4.26-7.94	.001	22.3	18.7-27.3	22.5	14.2-27.6	
<i>Hemoglobin, gm/100 cc.</i>						<i>Reticulocytes, %</i>				
1	12.1	11.0-13.0	11.8	11.0-12.5		0.6	0.3-0.9	1.3	0.6-2.1	.05
2	10.8	10.0-11.5	12.4	10.5-14.0	.001	1.0	0.4-1.7	2.6	0.4-6.4	.001
3	10.6	9.8-12.0	11.7	10.0-12.8	.001	0.9	0.6-1.5	1.7	0.6-4.0	.01
4	11.4	10.8-11.8	11.6	11.2-12.0	.2	1.2	0.7-1.7	1.8	1.5-2.2	.01
5	13.7	12.4-14.4	14.3	14.5-15.2	.1	1.0	0.8-1.2	1.5	0.7-2.6	.1
6	12.0	11.6-12.4	12.2	11.8-12.6	.2	0.8	0.4-1.4	1.3	0.8-2.0	.05
Av.	11.7	9.8-14.4	12.3	10.0-15.2	.001	0.9	0.3-1.7	1.7	0.4-6.4	.001

*p* calculated according to Fisher. Considered significant when .05 or less.

averages for the experimental values ranged from 1.3 to 2.6 per cent with a mean of 1.7. Individual values up to 6.4 per cent were found during cyanide administrations.

The red cell diameter measurements did not show a statistically significant change. The mean values for 100 cell diameters of each of 3 rabbits during the control period were 6.7, 6.8, and 6.8µ. During the experimental period, the corresponding values were 7.2, 7.3, and 7.4µ.

## DISCUSSION

Increases in red cell count and hemoglobin values which were produced by cyanide administration were considerably less than those which have been produced by anoxic anoxia. There are several possible reasons for this. In the first place, it is

probable that the duration of severe anoxia from a single dose of cyanide per day is rather brief in any one 24-hour period, since detoxification starts immediately, and the rabbit, according to Horten (6), is able to detoxify 27 $\mu$ g. of NaCN per kg. of body weight per minute. Since the rabbits used in our work were able to tolerate increasingly large doses of cyanide, it may be that their ability to detoxify the substance increased, although it does not seem likely that this accounts for all of the increased tolerance. Horten believes that the rate of detoxification is nearly constant for all rabbits, so that what may be called by the rather vague term 'acclimatization' is at least partly responsible. In other words, the animal is able to carry on its bodily functions in the presence of increasingly large concentrations of cyanide in the tissues.

Furthermore, cyanide has been shown to have effects other than those produced by the blocking of the respiratory enzyme. It was shown by Loevenhart (7) that in small doses, cyanide acts as a stimulant to the respiratory center, independent of any change in the circulation. Evans (8) also demonstrated this and reported stimulation of the vasomotor and cardio-inhibitory centers of the medulla. Large doses caused depression of these centers. In the present experiments, the animals apparently received a dose only strong enough to stimulate the medullary centers, since a depression in the respiratory rate was seen in animals only just before death.

Cyanide might tend to limit the increase in the red cells by altering the respiratory rate of the marrow. However, the evidence justifies the conclusion that cyanide administration causes a genuine stimulation of the erythropoietic function of the bone marrow. Krumdieck (9) reports that the normal reticulocyte count in rabbits is between 0.5 and 2.0 per cent and rarely rises over 3.0 per cent. Cyanide administration repeatedly produced values over 3 per cent, and the increases were statistically significant.

The results obtained in the experiments reported here showed a tendency for the diameter of the red cells to increase; however, the change was not statistically significant. With anoxic anoxia, Dubig (5) reported a slightly increased diameter, diminished mean corpuscular hemoglobin, and a slightly increased volume. Hurtado (4) reports that the natives at high altitudes show large erythrocytes. He found diameters of 8.04 $\mu$ , as measured on dried films. In this connection it should be noted that Ponder (10) states that the red cell diameter is 8 to 16 per cent less after being dried than while in serum or plasma.

It should perhaps be noted that in our series of rabbits the red cell counts and hemoglobin values were somewhat higher than those reported by Dill (11) for this animal. His values are: red cells, 4.55 million/mm.<sup>3</sup>, hemoglobin, 11.5 gm/100 cc. (derived from his value for oxygen capacity). This would make the mean corpuscular hemoglobin 25.2 $\mu$ g., a value higher than ours.

In conclusion, further work is indicated to learn if the other changes associated with adaptation to anoxic anoxia are also brought about by histotoxic anoxia and to learn whether or not there is any cross adaptation, that is whether or not an animal acclimatized to one type is acclimatized to other forms of anoxia.

#### SUMMARY

The effect of histotoxic anoxia on the red blood cells and hemoglobin was studied. After control data were obtained, daily subcutaneous injections of potassium cyanide



were administered. The mean values for the control periods for the 6 animals were: 5.12 million red cells/mm.<sup>3</sup>; 11.7 gm/100 cc. hemoglobin; and 1.0 per cent reticulocytes. Following administration of potassium cyanide, the results were: 5.59 million red cells/mm.<sup>3</sup>; 12.3 gm/100 cc. hemoglobin; and 1.7 per cent reticulocytes. The results were statistically significant. It is of interest that adaptive blood changes in rabbits can be stimulated by histotoxic anoxia as well as by anoxic anoxia.

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# INITIAL EPICARDIAL NEGATIVITY AND OTHER EXPERIMENTAL EVIDENCE RELATIVE TO VALIDITY OF ZONAL INTERFERENCE THEORY<sup>1</sup>

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PREVIOUSLY, (1, 2) we have shown that the rapid introduction of sufficient amounts of carbon dioxide (or of other dielectrics) into the right ventricle of dogs with open thorax results in the prompt and drastic reduction in size, or complete disappearance, of the initial upstroke in direct leads from the right ventricular epicardium. This change is often associated with a considerable increase in size of the S wave. We have presented evidence to show that this alteration in QRS complex is not due to anoxia, to right ventricular dilatation or to change in blood pH. Attempts to explain the phenomenon on the basis of the existing dipole hypotheses have failed. We have turned therefore to the 'zonal interference' concept of electrocardiographic genesis (3-5) and have tested this theory in the light of our findings. According to this concept, the electrocardiogram in any lead is the result of the interference between effects produced by a zone proximal to, and those produced by a zone distal to the exploring (positive) electrode. The proximal zone contribution to the QRS complex is believed, according to this hypothesis, to be a downward movement of the beam (QS), and the distal zone contribution an upward movement (R). Proximal areas are found to be large in unipolar limb leads (3), and much smaller in precordial derivations (4). It is possible, therefore, to suppose that the area of the proximal zone in *direct* epicardial leads would be even smaller. If this were found to be so, then the elimination of the conductivity effects of right ventricular blood (6) by its replacement with a dielectric could be assumed to result in electrical isolation of the zone of right ventricular muscle in contact with the exploring electrode from more distant parts of the heart. Proximal zone effects therefore would not be opposed by distal zone effects, and the major deflection of the beam would be a downward one. This would explain the disappearance or marked reduction in the size of the R wave (distal zone effect) shown to occur under these circumstances, and it would explain also the increased size of the S wave (proximal zone effect). It is to be noted that, because the thorax is open, the anterior epicardial surface, other than under the electrode, is air insulated.

In the experiments reported the first objective was to determine the size of the proximal zone by the induced extrasystole method in direct leads from the canine epicardium. The second objective was to determine whether or not with spontane-

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ously occurring systoles a proximal zone effect could be demonstrated. It was assumed in connection with the latter objective that, if such a proximal zone exists for spontaneous systoles, then it should be located at the site of earliest activation of the epicardial surface. According to the hypothesis of Nahum and Hoff such a proximal zone should yield complexes showing primary negativity.

#### METHOD

Dogs, with open chest via a mid-line sternum splitting incision, were the test objects. Anesthesia was obtained with pentothal 20 mg/kg. and barbitol 150 mg/kg. by vein.

*Area of Proximal Zone of Direct CV Leads.* A wick electrode, having an area of contact of 0.5 sq. cm., was placed lightly in contact with the anterior surface of the right ventricle as the exploring (positive) pole of a CV lead. Simultaneous CV and  $V_R$  leads were recorded. Forced mechanical epicardial extra-systoles were induced in a manner previously described (1). These extrasystoles were first induced at the area of application of the wick, and then at varying distances from it in order to outline the extent of the proximal and distal zones in accordance with the technique of Nahum and Hoff.

*Location of the Area of Primary Activation (Negativity) on the Epicardial Surface.* In most cases the pericardium was opened through a small slit along the interventricular septum. In 2 cases the pericardium was intact. Needle electrodes, as direct CV leads, were placed in the areas indicated by Lewis and Rothschild (7), Nahum *et al.* (3), Harris (8) and others, moving them a millimeter at a time until a primary negative deflection was obtained. These points were followed on the cathode ray oscillograph and when the desired points were found, records were made simultaneously on string galvanometers connected to the animals. Standard or unipolar leads were also recorded as controls. The needles were checked as to depth of penetration so that there was no possibility of picking up cavity potentials.

#### RESULTS

Extrasystoles induced at the site of the wick electrode placed on the mid-anterior right ventricle (by stimulating mechanically directly between the strands in contact with the epicardium) were always downward deflections of the beam (QS). Extrasystoles at points removed from this area by more than 0.5 cm. in any direction always gave an initial positive (upward) deflection followed by a downward one (RS). As the distance from the area of the direct electrode increased, the R wave increased in height in the forced mechanical epicardial premature contractions. Simultaneously recorded extrasystoles in  $V_R$  leads remained negative as their point of origin was moved upward from the direct wick electrode to the pulmonary conus. They also remained negative as their site of origination moved downward. When the right and left apex was reached the extrasystoles in  $V_R$  became positive (fig. 1) while the CV lead still showed an RS.

The point of initial negativity (PIN) was first sought using a CV wick electrode. This proved unsuccessful since a preliminary R wave was always present. When CV needle electrodes were used a PIN was located in 11 out of 14 experiments. In

3 cases a small initial R persisted. In one case two PIN were found. This point was always to the right of the septum near the edge of the trabeculated area described

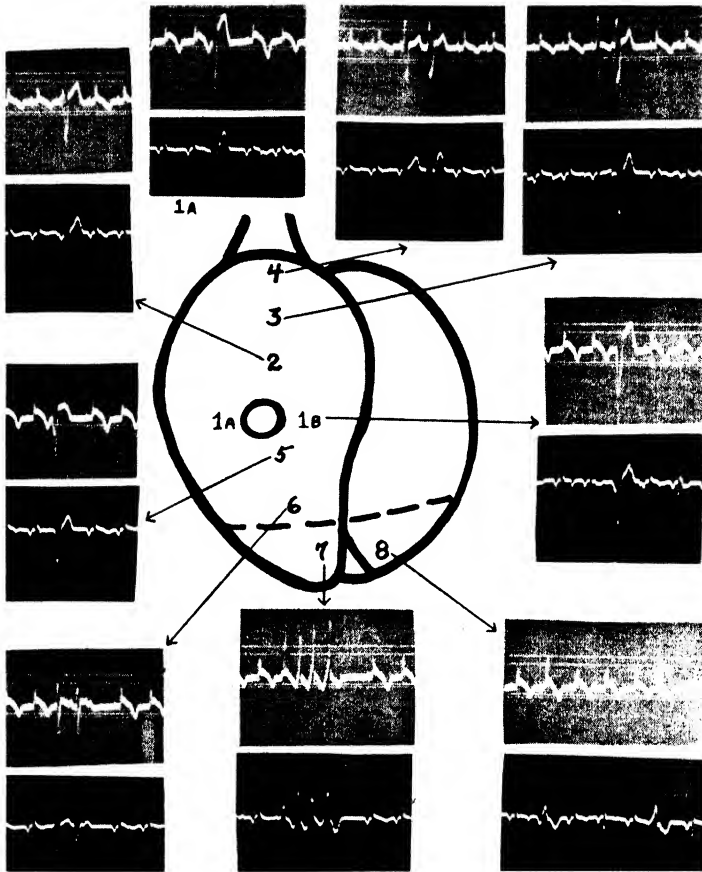


FIG. 1. MECHANICALLY INDUCED EXTRASYSTOLES. Wick electrode for CV lead located on epicardial surface of canine right ventricle at spot marked by a circle. Numbers indicate sites stimulated mechanically. At 1.1 the stimulation was accomplished by introducing the needle through the center of the wick electrode. The electrocardiograms obtained for this point of stimulation are in the second vertical row at the top. Arrows indicate electrocardiograms for other sites. In each instance the CV lead is at the top, the simultaneously recorded  $V_R$  is below. The interrupted line extending across both ventricles near the apex divides the proximal zone for  $V_R$  (above) from the distal zone for  $V_R$  (below). It will be noted that the extrasystoles in  $V_R$  derived from proximal zone stimulation are negative deflections, while those derived from distal zone stimulation are positive. The proximal zone for the direct lead, on the other hand, is limited to a very small area at the site of the wick electrode. Points 1A and 1B were stimulations at the wick contact site (proximal zone). Point 5 was close enough to be in the proximal zone. Camera speed 25 mm/second. String galvanometers.

by Lewis (7) (fig. 2). When PIN was found the complete absence of any preliminary positive wave was checked by increasing the amplitude of the trace on the monitor-

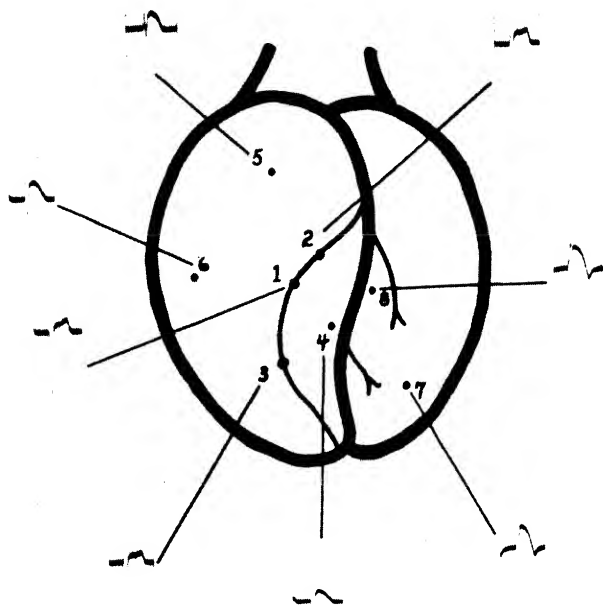


FIG. 2. DIRECT CV LEADS from anterior surface of canine heart. Sites at which needle electrode was successively located are indicated by *numbered dots*, and the corresponding electrocardiograms are indicated. The point of initial negativity (PIN) is located at 1. Moving the electrode a short distance away from this point (as at 2) resulted in the appearance of an initial positive deflection. *Curved line* on right ventricle is right edge of the trabeculated area (7). Camera speed 25 mm/second. Consolidated type 7-115 d'Arsonval galvanometers.

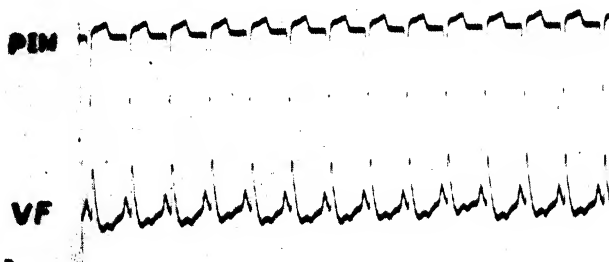


FIG. 3. SIMULTANEOUSLY RECORDED DIRECT LEAD at PIN and leaf VF. None of the complexes obtained at PIN show any sign of initial upward deflection of the QRS complex. VF shows that PIN is in distal zone for that unipolar lead. Recording same as in figure 2.

ing cathode ray oscillograph at least two-fold. Deviation from this point in all directions by more than 5 mm. produced an initial upward deflection. Standard and uni-

polar leads recorded at the same time were non-contributory (fig. 3). Points on the endocardial surface simultaneously recorded beneath the epicardial PIN were likewise negative.

#### DISCUSSION

Direct epicardial leads have small proximal zones as indicated by the restricted areas giving downward beam movements with forced mechanical extrasystoles. No evidence of intermediate zones was obtained. Distal zones were large as judged from the extensive areas giving upward deflections. A similar result was obtained in a chart published to illustrate patterns of ventricular extrasystoles (5). With a direct CV lead on the left apex extrasystoles elicited at mid-right lateral ventricle, mid-anterior septum, mid-left lateral ventricle, and mid-posterior septum all gave an initial upward beam movement (distal zone). However, those elicited at the left apex (proximal zone) gave an initial negative deflection (5).

The PIN was located at that point which previous work had indicated to be the site of earliest epicardial excitation, viz. to the right of the septum near the right edge of the trabeculated area (7), at the junction of its upper and middle thirds. This point was very restricted. PIN is a downward beam deflection since, when the excitation breaks through to the epicardial surface at this point, it is proximal to all other epicardial surfaces. This represents a pure type of intrinsic deflection because the area of activity and contact are the same. A similar statement could be made concerning extrasystoles induced at the area of the wick electrode. It is difficult to explain a PIN on a cavity potential hypothesis. The PIN is most often a pure QS with no R wave at all following the initial downward movement of the beam. The PIN was located, and the findings were the same when the pericardium was closed around the exploring electrode, the pneumothorax reduced, the chest closed and spontaneous respiration allowed to resume.

Thus it may be suggested that when dielectrics are placed in the right ventricle, eliminating the contribution of the contained blood (6) and the overlying myocardium (1), any direct epicardial lead will record only from its immediate zone of contact (proximal). As a result, the decrease in R wave and increase in S wave, in direct leads from the anterior right ventricle surface when dielectrics fill the right ventricle may be explained as due to the elimination of distal zone effects. In the absence of dielectrics, direct CV leads from the epicardium, except at PIN, give initial positive deflections since they are always in the distal zone at the beginning of spontaneously occurring systoles. They are not proximal effects as was first thought (1).

Wick electrodes apparently cover too large an area to demonstrate a PIN in most cases. Because of the small proximal area for PIN the wick electrode probably included both proximal and greater distal areas, and resulted in our inability to eliminate a small initial positive deflection with this type of electrode. When resort was had to needle electrodes with small areas of contact PIN could be demonstrated.

#### SUMMARY

Mechanically induced extrasystoles in dogs result in downward movements of the electrocardiographic beam (QS) in direct leads from the site of initial excitation

and from a very small surrounding zone. In more distant areas an upstroke results. These findings are in accord with the Nahum and Hoff proximal-distal zone interference concept, and indicate that the size of the proximal zone is even smaller in direct leads than it is in leads from the precordium.

Direct leads by means of a needle electrode make possible in most instances the discovery of a very limited area on the epicardial surface of the canine right ventricle from which QS deflections are obtained. This point of initial negativity (PIN) is located to the right of the septum near the right edge of the trabeculated area, at the junction of its upper and middle thirds, and corresponds to the site considered to be that of earliest activation of the epicardial surface. It appears, then, that a proximal zone effect may be demonstrated for spontaneously occurring ventricular excitation as well as has been done for induced extrasystoles. This phenomenon of initial epicardial negativity is difficult to explain on the basis of the classical dipole hypothesis of electrocardiographic genesis.

The findings in these experiments provide a possible explanation for the disappearance or drastic reduction in size of the R wave in direct leads from the right ventricular epicardium when the right ventricular cavity contains a dielectric and the chest is open. The very small zone in contact with the electrode is electrically isolated by the dielectric from distal areas of the heart, and one would expect therefore to derive a predominantly proximal (QS) effect.

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# EFFECT OF AURICULAR FIBRILLATION ON CARDIAC OUTPUT, CORONARY BLOOD FLOW AND MEAN ARTERIAL BLOOD PRESSURE<sup>1</sup>

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THE effect of auricular fibrillation on the mean coronary blood flow and arterial blood pressure was reported in a previous paper (1). Certain definite conclusions were reached, but a complete interpretation of the phenomena observed proved impossible because no simultaneous measurement of cardiac output was available. Recently a new technique has been developed which, through the use of a rotameter, allows continuous recording of the output of the left ventricle (2). By employing simultaneously one rotameter to record the coronary flow as reported previously (1, 3), and another rotameter to record the output of the left ventricle as described in the newly designed technique (2), it has become possible to observe the effect of auricular fibrillation on the cardiac output, coronary blood flow and mean arterial blood pressure simultaneously. It is the purpose of the present paper to report the results of these studies.

## METHOD

Nine dogs weighing from 18 to 27 kilograms were used. They received intravenously 0.4 to 0.5 cc. of a 6 per cent sodium pentobarbital solution per kilogram of body weight. The chest was opened through a midsternal incision and, under artificial respiration, the heart was suspended in a pericardial cradle. The portion of the left subclavian artery near the aorta, the brachiocephalic trunk, a portion of the two common carotid arteries in the lower part of the neck and a segment of the descending aorta distal to the left subclavian artery were dissected. A segment of the left anterior descending coronary artery was also dissected (1). The blood was then rendered incoagulable by the intravenous administration of an initial dose of 5 mg. heparin per kg. of body weight, then 3 mg. per kg. every half hour. As seen in figure 1, the aortic end of the left subclavian artery *A*, was cannulated and connected to the inflow tube *D* leading into the flowmeter (rotameter) *F*. The cephalic

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<sup>5</sup> Fellow of the American Heart Association.



ends of the two common carotid arteries were then cannulated successively and connected to the outflow tube *E* leaving from the rotameter *F*. At this stage of the operation, the blood flowed from the aorta into the cephalic ends of the two common carotid arteries *C* via the left subclavian artery *A*, the inflow tube *D*, the shunt *B* and the outflow tube *E*. Then the aortic end of the brachiocephalic trunk *K* was cannulated and connected to the inflow tube *D*. Following this, the descending aorta was ligated at *G* just beyond the left subclavian artery and its peripheral end was cannulated and connected to the outflow tube *E*, at *H*. In this manner, except for the blood entering the coronary arteries and other small arteries originating from the aorta between the aortic valves and ligature *G*, the total output of the left ventricle flowed into the two common carotid arteries and the upper part of the descending aorta via *A* and *K*, *D*, *B* and *E*. When shunt *B* was later occluded, the

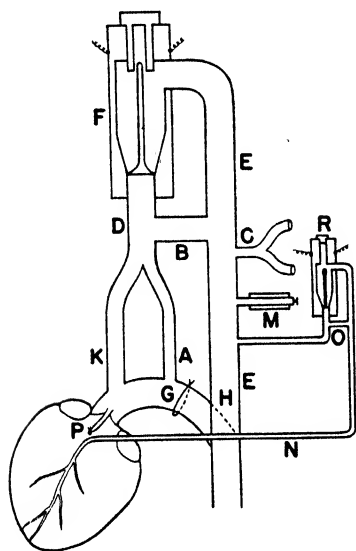


FIG. 1. SCHEMA of apparatus used to record simultaneously and continuously the output of the left ventricle, mean coronary blood flow in the left anterior descending coronary artery and mean arterial blood pressure.

left ventricular output flowed through the rotameter *F*. Finally, the left anterior descending coronary artery was ligated at *P* and its distal portion cannulated and connected to the outflow tube *N* coming from the rotameter *R* used to measure the blood flow through this coronary artery. Shunts *B* and *O* were left open whenever a zero flow was desired for the cardiac output meter and the coronary flow meter respectively, without interrupting the flow of blood and they were occluded in order to record the output of the left ventricle and the coronary blood flow. The flow meters were calibrated as previously described (3). An optical manometer *M* was used to record the mean arterial blood pressure.

Auricular fibrillation was induced by electrical stimulation of minimal intensity, applied to the right or left auricular appendage through two fishhook electrodes 0.5 to 1.0 cm. apart. An electrocardiogram was recorded at appropriate intervals.

## RESULTS

Thirty-four bouts of auricular fibrillation induced in 9 dogs were studied. Electrical stimulation was applied for from 38 to 300 seconds. In some cases, fibrillation ceased as soon as stimulation was discontinued, and in other cases it persisted for some time after stimulation was discontinued. No difference in the pattern of the cardiovascular reactions observed was found between the bouts of fibrillation maintained by continuous stimulation and those that persisted after stimulation was discontinued. When the operative procedure was finished, 2 of the 9 dogs were observed to have mechanical alternation of the heart, although their blood pressure and cardiac output were satisfactory. These 2 dogs received intravenously, one 0.4 mg. and the other 0.8 mg. lanatoside C. The cardiovascular reactions observed in these 2 dogs were indistinguishable from the phenomena observed in those dogs which did not receive lanatoside C. The effect of auricular fibrillation on cardiac output, coronary blood flow and arterial blood pressure was essentially the same in

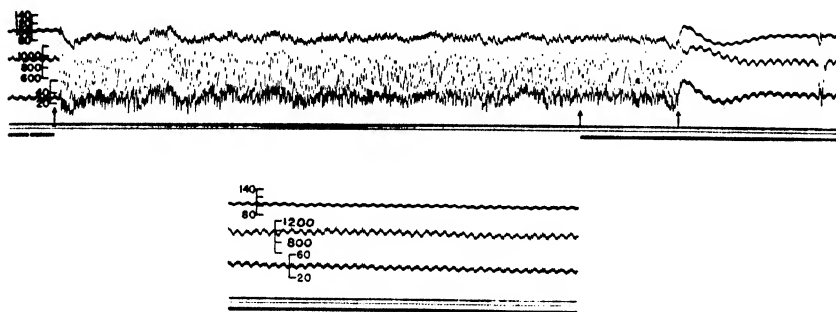


FIG. 2. BOUT OF AURICULAR FIBRILLATION. *Upper tracing*, Mean arterial blood pressure; scale in mm. Hg. *Middle tracing*, mean output of left ventricle; scale in cc. per minute. *Lower tracing*, mean blood flow in left anterior descending coronary artery; scale in cc. per minute. Time in seconds. At *first arrow*, beginning of electrical stimulation and induction of auricular fibrillation. At *second arrow*, end of electrical stimulation. At *third arrow*, end of auricular fibrillation.

all experiments and is illustrated by the record reproduced in figure 2. During the control period, the arterial blood pressure oscillated around 102 mm. Hg and the cardiac output around 950 cc. per minute. The coronary blood flow fluctuated around 30 cc. per minute and the heart rate was 180 per minute. As soon as electrical stimulation was applied, auricular fibrillation resulted; immediately arterial blood pressure, cardiac output and coronary blood flow fell markedly. Because of the inertia of the rotameters, it is not possible to measure exactly the cardiac output and coronary blood flow during the bout of auricular fibrillation. However, it seems that during the bout of auricular fibrillation, the coronary flow was often above its control value and that the cardiac output was below its control value throughout the bout of auricular fibrillation. The arterial blood pressure also remained below its control level throughout the bout of auricular fibrillation. During auricular fibrillation, the ventricular rate varied between 220 and 230 per minute. Electrical stimulation was maintained for 175 seconds, after which auricular fibrillation per-

sisted for about 32 seconds. As soon as auricular fibrillation stopped, blood pressure, cardiac output, and coronary flow rose and reached a maximum of 122 mm. Hg, 1265 cc. per minute and 63 cc. per minute, respectively. Then all three decreased. The blood pressure reached a minimum of 80 mm. Hg and the coronary flow a minimum of 29 cc. per minute. It was observed very frequently that blood pressure and coronary flow decreased more markedly than the cardiac output at this period and that their tracings were quite parallel. Then blood pressure and coronary flow rose and oscillated around a maximum of 102 mm. Hg and 36 cc. per minute, respectively. The cardiac output during this time kept decreasing progressively and at the time the blood pressure oscillated around 102 mm. Hg and the coronary flow around 36 cc. per minute, the cardiac output was 950 cc. per minute. One hundred and sixty-four seconds after the end of auricular fibrillation, both blood pressure and cardiac output had returned to their control levels, i.e. 102 mm. Hg and 950 cc. per minute, whereas the coronary flow was still 37 cc. per minute. Seven minutes after the end of fibrillation, although the blood pressure was 98 mm. Hg, a value

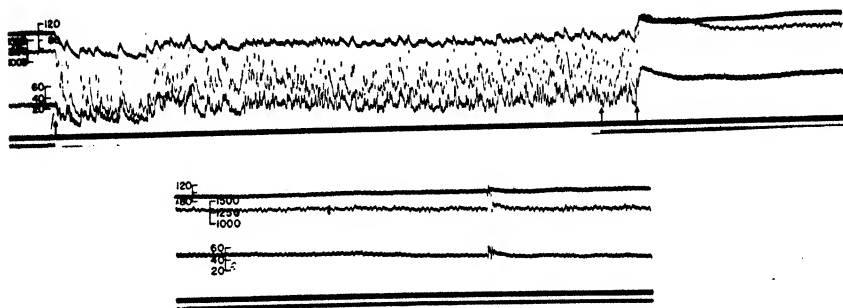


FIG. 3. BOUT OF AURICULAR FIBRILLATION. Same legend as in figure 2.

slightly below control, and the cardiac output was 840 cc. per minute, a value definitely below control, the coronary flow still amounted to 32.5 cc. per minute, a value slightly above control.

Figure 3 is the record of a bout of auricular fibrillation induced in another dog. It illustrates essentially the same cardinal features as the bout of figure 2, but also demonstrates a few minor variations that were occasionally encountered. During the bout of fibrillation, the arterial blood pressure remained far below its control level and the coronary flow was very frequently below its control; when fibrillation stopped, the blood pressure increased above its fibrillation level but did not rise above its control level; finally, during recovery from fibrillation, never did blood pressure or coronary flow decrease very markedly as seen in the bout of figure 2.

#### DISCUSSION

Certain definitive conclusions can be drawn from the reported studies. Thus, at the onset of auricular fibrillation, the coronary flow decreases. This decrease is presumably due to the marked drop in arterial blood pressure produced by the marked

drop in cardiac output, which occurs at the onset of fibrillation. Although, because of the inertia of the rotameters, no accurate values of the coronary flow and cardiac output are available during auricular fibrillation, it is clear that after 5 or 6 seconds of fibrillation, arterial blood pressure, cardiac output, and coronary blood flow increase. The increase in cardiac output is probably due to myocardial adjustments and possibly to extra-cardiac adjustments, the nature of which is not obvious from these experiments. Whether the increase in arterial blood pressure toward control level, which occurs after a few seconds, is due to the increase in cardiac output alone or whether it is partly due to an increase in cardiac output and partly due to peripheral vasoconstriction, cannot be ascertained from the experimental data at hand. Since, however, after the first few seconds of auricular fibrillation, the coronary flow rises and often reaches and remains above its control level, whereas arterial blood pressure and cardiac output are below their control levels, it must be concluded that during auricular fibrillation the resistance of the coronary bed is decreased. Whether this decrease in the resistance of the coronary bed is due to active dilatation of the coronary bed or due to a decrease in the extravascular support of the coronary bed, or both, is uncertain.

As fibrillation stops, arterial blood pressure, cardiac output, and coronary blood flow increase and rapidly reach a peak. Calculations of the total peripheral resistance at this peak show that the increase in the mean arterial blood pressure is due exclusively to the increase in cardiac output. In the experiment shown in figure 2, for example, the control total peripheral resistance<sup>6</sup> is 8581 units and when the cardiac output is at its peak, it is 7708, indicating that at this moment the blood pressure is elevated because of the increase in cardiac output and despite some peripheral vasodilation. At this time of maximal increase in cardiac output, there was a decrease in the total peripheral resistance in all except one bout of fibrillation in which there was a slight increase of the peripheral resistance. The concomitant increase in coronary blood flow can be accounted for by the increase in the aortic blood pressure and/or by the increase in the work of the heart. Whether at this point there is also a decrease in the peripheral resistance of the coronary bed cannot be ascertained. No attempt was made to solve this point by calculating the peripheral resistance of the coronary bed with the formula used above for the calculation of the systemic total peripheral resistance because the value of such a method in the case of the coronary circulation is open to question. After a peak is reached by arterial blood pressure, cardiac output and coronary flow, all three decrease. In most cases, blood pressure and coronary flow decrease rapidly and within 10 to 15 seconds after the end of fibrillation, both reach a minimum which may be below their respective control values. Then both rise again and reach a second maximum, after which they both return progressively to control values. The cardiac output, after reaching its peak, comes down progressively to its control level. In a few of the shortest bouts of auricular fibrillation, arterial blood pressure, cardiac output, and

<sup>6</sup> The total peripheral resistance was calculated from the formula:

$$T.P.R. \text{ (dynes cm.}^{-5} \text{ sec.)} = \frac{(\text{mean arterial blood pressure in mm. Hg} \times 1332 \times 60)}{\text{cardiac output in cc/min.}}$$

coronary flow resume their control values rapidly and simultaneously. In most experiments, however, arterial blood pressure and cardiac output return to their control level before the coronary flow (figs. 2, 3). This persistent increase in the coronary flow must be due to a decrease in the peripheral resistance of the coronary bed. Whether this is due to actual vasodilation of the coronary bed or to a decrease of the extravascular support of the coronary bed is not known. It seems more probable, however, that the metabolites that have accumulated in the myocardium during the bout of fibrillation are responsible for a persistent vasodilation of the coronary bed.

#### SUMMARY

Thirty-four bouts of auricular fibrillation electrically induced in nine dogs were studied as to their effect on the mean arterial blood pressure, cardiac output and coronary blood flow. At the onset of fibrillation, there is an abrupt and marked fall in blood pressure, cardiac output, and coronary flow. After a few seconds there is some rise in all three toward control values, the coronary flow rising in most experiments at least temporarily above its control level. At the termination of fibrillation, there is a sudden increase in all three to levels greater than control, except for an occasional experiment in which the blood pressure rises but not to or above its control level. Following some of the short bouts of fibrillation, arterial blood pressure, cardiac output, and coronary flow return to control rapidly and simultaneously. In most of the bouts of fibrillation, the coronary flow remains above control level after cardiac output and arterial blood pressure have returned to control values. The mechanism of the cardiovascular reactions observed has been discussed.

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# EVIDENCES FOR MORE THAN ONE ANTIDIURETIC SUBSTANCE IN PITRESSIN<sup>1</sup>

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IN THE course of experiments on the antidiuretic substance extractable from urine (1-3), we have simultaneously assayed pitressin, treated in several ways, in dogs and rats. The observations have revealed an interesting species difference.

## EXPERIMENTAL PROCEDURE

*Rat Assays.* Antidiuretic assays on rats were made by the method of Burn (4). Male rats weighing on an average of 200 gm. were deprived of food and water for 15 hours prior to the test. A total of at least 3 cages of 4 rats each was used for each assay and at least 3 separate assays were made on each substance to be tested. Each rat was hydrated by gavage to 5 per cent of its body weight with tap water at 37° C. The substance to be assayed was injected intraperitoneally. Rats in groups of 4 were placed in a metal cage set in a large glass funnel and the urine was collected directly into volumetric cylinders. The volume of urine excreted was noted at 15-minute intervals starting 30 minutes after hydration was begun and continuing until 50 per cent of the ingested water had been excreted or for 240 minutes if 50 per cent excretion was not achieved. Chloride determinations were by the Van Slyke modification of Sendroy's method (5) on urine samples representing 25 per cent of the ingested water.

*Dog Assays.* The dog assays were made on normal unoperated female dogs who were either trained and unanesthetized or were under light chloralose anesthesia. Water diuresis was established by administering by gavage 75 to 150 cc. of water per kg. of body weight. When the urine flow was greater than 3 ml./minute the material to be tested, diluted to hypotonicity with respect to plasma, was injected i.v. through an indwelling needle into the jugular or forearm vein. Urine was collected from an indwelling 'mushroom' catheter at intervals of 5 to 20 minutes. Injections of freshly diluted commercial pitressin (Parke, Davis and Company) were given during the assay experiments in order to quantitate the antidiuretic potency of the unknown substance being tested. Pitressin in doses of 2 to 20 mU gave distinct antidiuretic effects, depending upon the initial rate of urine flow.

*Treatment of Pitressin for Assay.* Commercial pitressin (Parke, Davis and Company) was the source of pitressin in all experiments. The pitressin was assayed with-

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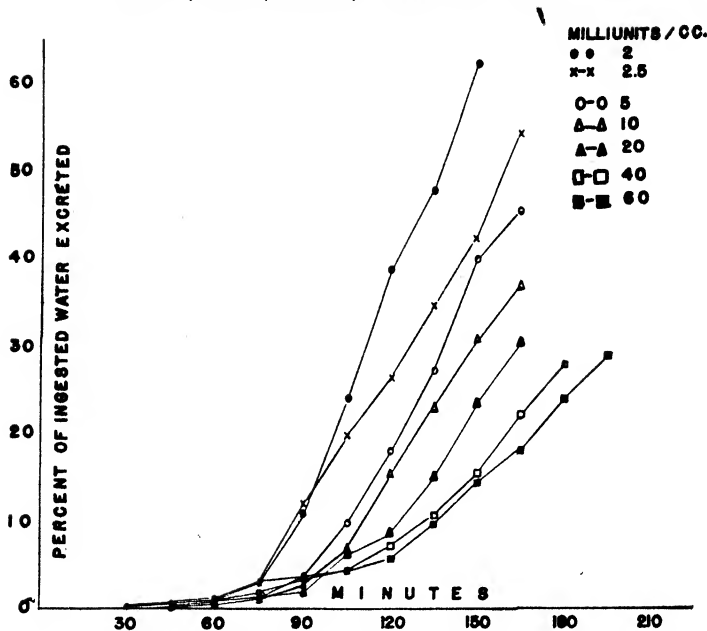


FIG. 1. RESULTS OF ASSAYS of different doses of pitressin in hydrated rats.

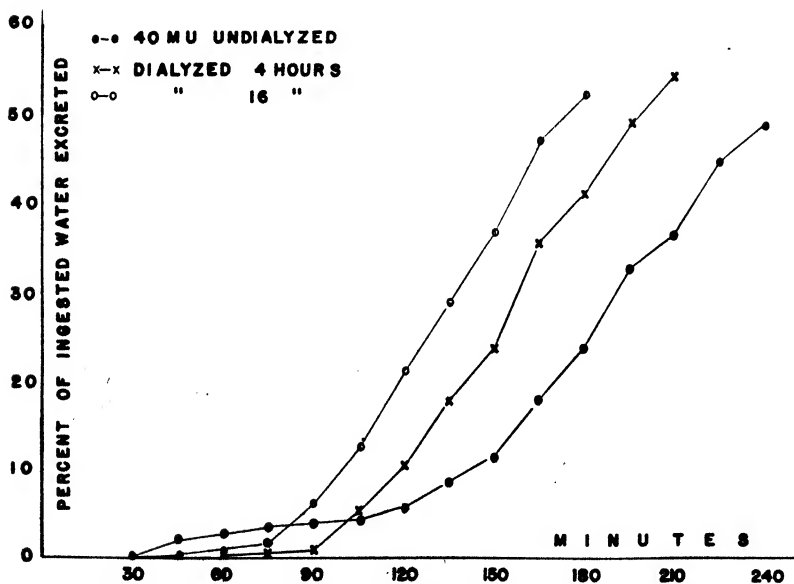
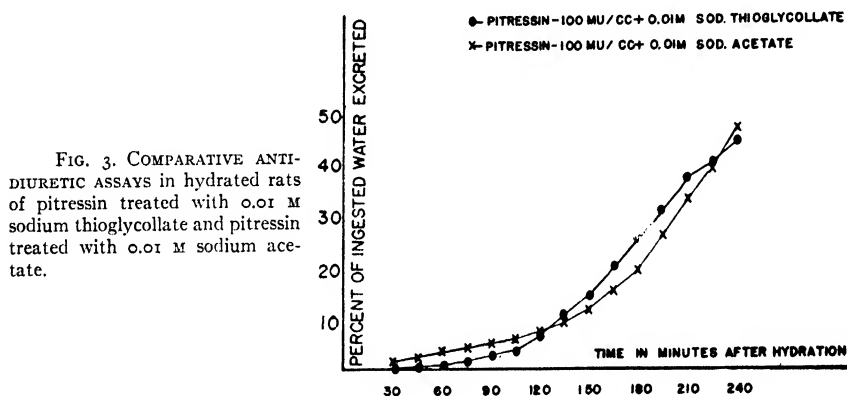


FIG. 2. EFFECT OF DIALYSIS on the antidiuretic activity of pitressin assayed in hydrated rats.

out any treatment in varying doses in both dogs and rats. Commercial pitressin, dialyzed in Visking cellulose sausage casing 36/32 for  $\frac{1}{2}$ , 4 and 16 hours in running water, was tested in both dogs and rats. In each instance controls were tested with the same lot of undialyzed pitressin in the same doses, as it was observed that the antidiuretic effect of pitressin varies somewhat in different lots. Pitressin treated with 0.01 M sodium thioglycollate as described by Ames *et al.* (6) was assayed in dogs and rats. As a control in these assays pitressin treated with 0.01 M sodium acetate was injected into both dogs and rats. This solution was adjusted to the same pH as the sodium thioglycollate solution.

### RESULTS

*Rat Assays.* Figure 1 shows the results of assays of untreated pitressin in doses ranging from 2 to 60 mU in hydrated rats. The results in these and in all the experiments in rats are expressed as the percentage of the ingested water excreted at 15-



minute intervals for the period of the test. The results are confirmation of the method of assay reported by Burn (4) and show that the larger the dose of pitressin the greater the antidiuretic effect. We have reported previously (2) that the relationship of dose to antidiuretic effect is a logarithmic one.

Figure 2 shows the effects of dialysis on pitressin when assayed in rats; the dose of pitressin used in this experiment was 40 mU. The longer the time of dialysis the greater was the loss of antidiuretic potency *but* even after 16 hours some antidiuretic effect was still observed in the rats similar to the effect of about 2.5 mU of undialyzed pitressin (fig. 1). We have repeated these experiments many times and reported previously (1) that the antidiuretic potency of pitressin for rats did not decrease as rapidly as has been reported in assays of dialyzed pitressin in man (7).

Three separate experiments were done in rats with pitressin treated with 0.01 M sodium thioglycollate in the manner described by Ames, Moore and vanDyke (6). These experiments were done with different lots of pitressin and 9 cages of 4 rats each were used. A similar dose of pitressin treated with 0.01 M sodium acetate was



assayed as a control in 9 cages of 4 rats each. The results are shown in figure 3 and it is quite clear that sodium thioglycollate did not inactivate the antidiuretic effect of pitressin when assayed in rats.

*Chloruretic Effects of Pitressin, Dialyzed Pitressin and Pitressin Treated with 0.01 M Sodium Thioglycollate in Rats.* In all rat assays chloride was determined in the urine samples representing 25 per cent of the ingested water. The results are given in table 1. It was found that the chloride excretion following the injection of untreated commercial pitressin tended to increase as the size of the dose of pitressin was increased. As the urine samples represented 25 per cent of the ingested water and as the weight of the rats from cage to cage did not vary more than 5 per cent, the volumes of urine on which the chloride was determined were similar. When the micro-equivalents of chloride excreted per minute are plotted against the log dose of pitressin (fig. 4) a relationship of antidiuretic to chloruretic effect is demonstrated for doses of pitressin

TABLE 1. CHLORIDE EXCRETION IN RATS FOLLOWING INJECTION OF PITRESSIN  
TREATED AS INDICATED

PITRESSIN DOSE	TREATMENT	MINUTES REQUIRED FOR 25% EXCRETION	CHLORIDE IN URINE REPRESENTING 25% EXCRETION
mU/cc.			mEq/l.
40	None	180	132
40	Dialyzed 4 hours vs. running tap H <sub>2</sub> O	150	66
40	Dialyzed 16 hours vs. running tap H <sub>2</sub> O	127	57
100	In 0.01 M sodium acetate	180	83
100	In 0.01 M sodium thioglycollate	160	45
100	In 0.01 M sodium acetate	186	113
100	In 0.01 M sodium thioglycollate	186	61
100	In 0.01 M sodium acetate	225	59
100	In 0.01 M sodium thioglycollate	201	32

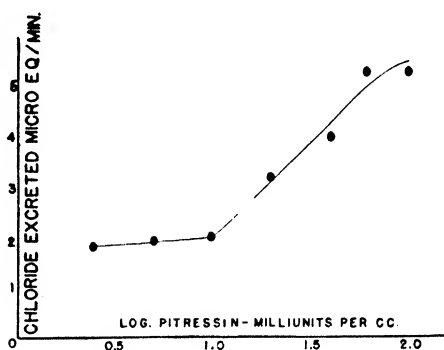
Brackets indicate different batches of pitressin.

ranging from 10 to 60 mU per cc. In doses less than 10 mU this relationship was not observed. Following dialysis the chloruretic effect decreased sharply. The results of dialysis are shown in table 1 and confirm our previous reports (1, 3) that dialysis decreases the chloruretic effect of pitressin when injected into rats. Similarly the chloruretic effect was decreased when pitressin was treated with sodium thioglycollate and under these circumstances, in each of the 3 experiments, chloride excretion was decreased to 55 per cent of that obtained with untreated pitressin.

*Dog Assays.* Pitressin dialyzed for  $\frac{1}{2}$ , 4 and 16 hours was assayed in the dog. A solution of 5 mU of pitressin per cc. dialyzed for  $\frac{1}{2}$  hour showed no appreciable loss of antidiuretic activity when compared with undialyzed material. Both the fresh and the dialyzed pitressin maintained urine flows of less than 2 ml/minute in doses of one cc. given every 15 to 20 minutes. When no pitressin was given the flow rose to more than 6 ml/minute. The pitressin dialyzed for 4 and 16 hours had an original concen-

tration of 40 mU per cc. After 4-hour dialysis considerable antidiuretic potency was still demonstrated, but the dialyzed pitressin did not depress urine flow as much as did undialyzed pitressin in similar doses. After 16 hours of dialysis the antidiuretic activity was so greatly reduced that one cc. of the solution originally containing 40 mU of pitressin had no more antidiuretic activity than did 4 mU of undialyzed pitressin. The effects of injecting pitressin treated with sodium thioglycollate are shown in figure 5. Water diuresis was established by giving 110 cc. of water per kg. of body weight by gavage, which in this dog amounted to 1500 cc. of water. Intravenous injections of thioglycollate-treated pitressin were alternated with injections of pitressin-sodium acetate. At the beginning of the experiment urine flow was increasing from 3.5 to 6.4 ml/minute. Pitressin thioglycollate in doses of 10 to 20 mU did not prevent this rise in flow. Untreated pitressin in doses of 0.5 mU showed a slight effect in the initial period of diuresis. When the urine flow had reached its peak 4 mU of untreated pitressin were required to inhibit diuresis. With a slowly falling urine flow doses of 50 and 100 mU of pitressin thioglycollate were less effective than were 2 mU of un-

FIG. 4. RELATION BETWEEN THE LOG DOSE of pitressin in milliunits and the chloride excretion in rats.



treated pitressin. The absolute effect of an antidiuretic material varies with the stage of water diuresis, but pitressin thioglycollate given in all stages and in doses ranging from 10 to 100 mU of original activity, had no antidiuretic effect in the dog. The same sample of pitressin thioglycollate assayed in rats showed no loss of antidiuretic potency.

#### DISCUSSION

These experiments suggest that the antidiuretic activity of commercial pitressin can be fractionated into more than one substance, one of which is active when assayed in the rat by the method of Burn (4) but not active when given i.v. to the hydrated dog. Other observers have reported that dialysis of pitressin results in a loss of its antidiuretic effect in man and in dogs (7). In our experiments dialysis of pitressin decreased the antidiuretic activity in both the dog and the rat assays and the extent of the loss of the antidiuretic effect depended upon the duration of the dialysis. In our experiments pitressin was dialyzed against running water as it was in the experiments of Donaldson (7) and Ham and Landis (8). Treatment with 0.01 M sodium thioglycol-

late as reported by Ames, Moore and vanDyke (6) abolished the antidiuretic effect of pitressin for dogs but had no effect on the fraction capable of producing antidiuresis in the rat.

The results also establish the fact that the chloruretic fraction of pitressin as tested in rats, is lost by dialysis and by treatment with thioglycollate. The dog assays were not designed to obtain data on chloride excretion but other observers have reported that commercial pitressin has a chloruretic effect in the dog and the rat (8, 9).

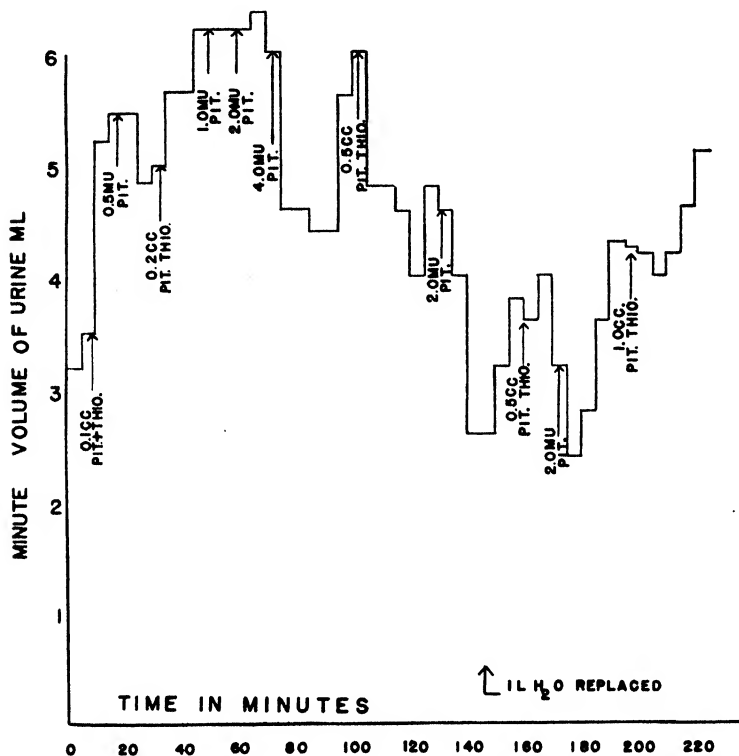


FIG. 5. ANTIDIURETIC EFFECT OF PITRESSIN treated with 0.01 M sodium thioglycollate and pitressin treated with 0.01 M sodium acetate injected i.v. into a female dog during water diuresis.

Judging from the assays in rats, the fraction responsible for chloruresis is destroyed by treatment that decreases or destroys the fraction antidiuretic for dog and man. We have reported previously (1) that 2-hour dialysis diminished the chloruretic effect of pitressin without affecting its antidiuretic effect as tested in rats. Donaldson (7) published pitressin assays in man presumably contradictory to our results following dialysis, but he failed to note the fact that we were speaking of assays in rats.

In previous studies using rat assays (1-3) we have reported that an antidiuretic non-chloruretic substance was extractable from the urines of normal dehydrated sub-

jects and from the urines of patients with cirrhosis of the liver. The urines in these assays were dialyzed for 2 to 4 hours to decrease the NaCl concentration. As the antidiuretic substance in urine has no chloruretic effect in rats, it may be that it is similar to the antidiuretic fraction still present in pitressin after dialysis or treatment with sodium thioglycollate. The maintained potency of thioglycollate-treated pitressin by the rat assay despite its inactivation for the dog, suggests a species difference in sensitivity to different antidiuretic material. It may be that the antidiuretic hormone contains more than one antidiuretic fraction and that these fractions can be separated by suitable treatment and demonstrated by assays in different species.

#### SUMMARY

Simultaneous assays of antidiuretic activity were made in dogs and rats with commercial pitressin, dialyzed pitressin and pitressin treated with 0.01 M sodium thioglycollate. Treatment with sodium thioglycollate abolished the antidiuretic activity of pitressin for the dog but had no effect on the antidiuretic activity in the rat. Dialysis of pitressin decreased the antidiuretic activity in both species. The longer the dialysis the greater was the loss of antidiuretic potency. The chloruretic effect of dialyzed and thioglycollate-treated pitressin was measured in the rat assays. Both procedures decreased the chloruretic effect significantly. The results suggest that the antidiuretic activity of commercial pitressin can be fractionated into at least two substances, one of which is active in the rat but not in the dog.

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# ACCELERATOR FACTORS IN HEMOPHILIC BLOOD

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THE fact that tissues or extracts of tissues accelerate the coagulation of blood *in vitro* has been long recognized, but the finding of thromboplastic substances in the blood plasma itself has been a more recent development. There are now a number of observations which seem to point to the existence of thromboplastins in plasma, thromboplastins that are not tissue derivatives, but that are associated with the plasma proteins (1-15).

During a series of experiments designed for the standardization of units of prothrombin (16), it was found that as prothrombin was purified, its rate of activation was markedly decreased, even when optimal amounts of calcium and rabbit brain emulsion were used, and that the defect could be corrected by the addition to the incubating mixture of a small amount of either normal serum, prothrombin-free plasma or 'euglobulin fraction' from normal serum. This decreased rate of activation of purified prothrombin and its acceleration by a substance, contained in the 'euglobulin fraction' of normal serum, formed a picture so similar to that of hemophilia that it suggested the possibility that we were testing for the same factor as the one described by Patek and co-workers (17-20). It was thought that if this were true, the activation of purified prothrombin would not be accelerated by hemophilic serum. The experiments reported herein are concerned with a comparison of the influence of normal and hemophilic sera on the rate of activation of purified human prothrombin, in the presence of optimal amounts of calcium and rabbit brain emulsion.

## REAGENTS

*Plasma.* Blood was collected from the antecubital vein as quickly as possible using a dry syringe and a 14-gauge needle. It was transferred to a test tube containing 0.1 cc. of 10 per cent potassium oxalate for every 10 cc. blood, and then centrifuged. The speed of centrifugation was not ascertained but the resulting plasma was clear and relatively free of platelets.

*Prothrombin-free Plasma.* One cc. colloidal  $\text{Al}(\text{OH})_3$  was added to each 10 cc. oxalated plasma; after standing 30 minutes it was rapidly centrifuged and the clear supernatant plasma used.

*Purified Prothrombin.* Purified prothrombin was prepared from human plasma using the method developed by Seegers (21), except that the procedure was carried out at room temperature and at a slower rate. The yields varied with each run, and were never as high as those reported by Seegers.

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**Serum.** Serum from human blood was used. Blood was allowed to clot and the serum, obtained by centrifugation, stood for at least one hour, or until all thrombic activity had disappeared.

**Colloidal Aluminum Hydroxide.** Colloidal  $\text{Al}(\text{OH})_3$  was prepared by the method used by Quick (22) in preparing  $\text{Mg}(\text{OH})_2$ .

**Calcium Nitrate.** From a N/10 stock solution of  $\text{Ca}(\text{NO}_3)_2$ , solutions of the desired strength were prepared as needed with buffered saline.

**Buffered Saline.** Ten cc. imidazole buffer was added to each 90 cc. of 0.9 per cent NaCl solution.

**Imidazole Buffer.** Imidazole buffer was prepared by the method of Mertz and Owen (23).

**Thromboplastin.** The thromboplastic material employed throughout the experiments was freshly prepared rabbit brain emulsion prepared by a technique similar to that described by Quick (24).

TABLE 1. ACCELERATING EFFECT OF HEMOPHILIC SERUM ON THE ACTIVATION OF PURIFIED HUMAN PROTHROMBIN

EXPER. NO.	PURIFIED HUMAN PROTHROMBIN	RABBIT BRAIN EMULSION	CALCIUM CHLORIDE SOLUTION (0.5%)	NORMAL SALINE	SERUM	INCUBATION TIME	THROMBIN DEVELOPED
	cc.	cc.	cc.	cc.	cc.	sec.	units
a	0.5	0.25	0.25	0.1	o	60	0.06
b	0.5	0.25	0.25	o	Normal 0.1	60	17.88
c	0.5	0.25	0.25	o	hemoph. <sup>1</sup> 0.1	60	17.88
d	0.5	0.25	0.25	o	hemoph. <sup>2</sup> 0.1	60	17.88

<sup>1</sup> 12-year old male, bleeding (Charles C.).      <sup>2</sup> 36-year old male, not bleeding (Malcolm C., brother of Charles C.).

**Visking Casing.** Visking casing no. 428, manufactured by the Visking Corporation, Chicago, was used as the dialyzing membrane throughout the experiments.

## RESULTS

**Comparison of Accelerating Effect of Normal and Hemophilic Sera on the Activation of Purified Human Prothrombin.** Purified prothrombin was incubated at room temperature with an optimal amount of calcium nitrate and rabbit brain emulsion in the presence of saline, normal serum or hemophilic serum. At appropriate intervals of time, 0.1-cc. samples were withdrawn from the mixtures and added to an equal volume of freshly prepared prothrombin-free plasma. The clotting time of the prothrombin-free plasma was used as a quantitative measure of the amount of thrombin developed. When it was desirable to convert the clotting time of the prothrombin-free plasma to percentage complete conversion, sampling was continued until there was no further evidence of thrombin formation. At this time the prothrombin was assumed to be 100 per cent converted. The results are summarized in tables 1 and 2. Repeated tests consistently showed that hemophilic serum was equally as effective as normal serum in accelerating the activation of purified prothrombin.

*Comparison of Effectiveness of Normal and Hemophilic Sera in Restoring the 'Prothrombin Time' of Stored Human Plasma.* In 1943 Quick (4) showed that when oxalated plasma is stored in the cold, the so-called 'prothrombin time' gradually increases, a fact which was interpreted by Quick (4) as an indication of the gradual disappearance of prothrombin from stored plasma due to oxidative processes. The prothrombin time of plasma from animals treated with Dicumarol is also markedly increased due to a reduction of the blood prothrombin (25). Quick (4) mixed plasma that had been stored in the refrigerator and fresh plasma from an animal which had received Dicumarol, and found that the resulting prothrombin time was lower than that of fresh plasma. This he interpreted as evidence that prothrombin is composed of 2 components, one of which, 'prothrombin A,' disappears when blood is stored, while the second, 'prothrombin B,' disappears when the animal is treated with Dicumarol. On the basis of work done in 1946 (5), however, Quick (6) aban-

TABLE 2. COMPARISON OF ACCELERATION OF ACTIVATION OF PURIFIED PROTHROMBIN BY NORMAL AND HEMOPHILIC SERUM

INCUBATION TIME OF MIXTURE	UNITS THROMBIN DEVELOPED		
	Prothrombin 0.5 cc. Rabbit brain emul. 0.25 cc. Ca(NO <sub>3</sub> ) <sub>2</sub> 0.25 cc. (M/200) Saline 0.1 cc.	Prothrombin 0.5 cc. Rabbit brain emul. 0.25 cc. Ca(NO <sub>3</sub> ) <sub>2</sub> 0.25 cc. (M/200) Normal serum 0.1 cc.	Prothrombin 0.5 cc. Rabbit brain emul. 0.25 cc. Ca(NO <sub>3</sub> ) <sub>2</sub> 0.25 cc. (M/200) Hemophilic serum 0.1 cc.
sec.			
60		4.96	4.52
120		6.96	6.04
240	3.48	9.10	8.08
360	3.76	11.28	
480		11.28	11.28
720	4.96	11.28	11.28
1080	5.52		
1680	6.04		
2100	8.08		
3600	9.16		

doned this view. 'Prothrombin B' was recognized as the true prothrombin, and 'prothrombin A' was found to be an accessory substance. The name 'labile factor' was substituted for 'prothrombin A.'

The question then arose as to the possibility that the defect in hemophilia might be an absence of this labile factor. With this in mind tests were performed to ascertain the effectiveness of dilute fresh hemophilic plasma in restoring the prothrombin time of stored plasma. Plasma was used instead of serum because of the difficulty in obtaining fresh non-thrombic serum from hemophilic blood.

Sterile human plasma with a prothrombin time of 15 seconds was stored in the refrigerator at 5° C. The one-stage prothrombin activity determination of Quick (26) was carried out. After 21 days' storage, the prothrombin time of the plasma was found to have increased to 68 seconds. The stored plasma was then mixed with an equal volume of either dilute fresh normal plasma (1:10 in saline) or dilute fresh hemophilic plasma (1:10 in saline). The prothrombin time of both diluted

plasmas was 50 seconds. The prothrombin time of each mixture was then ascertained. No significant difference was found in the effectiveness of either dilute fresh normal or dilute fresh hemophilic plasma in restoring the prothrombin time of stored plasma (table 3).

#### DISCUSSION

It seems probable that the labile factor of Quick (6), the accelerator factor of Fantl and Nance (8), factor V of Owren (9), Aeglobulin of Ware, Guest and Seegers (10-12), the accelerator factor of Mann, Hurn and Magath (15) and the plasma thromboplastin which we have described, are identical.

Hemophilic serum was found to be equally as active as normal serum in accelerating the activation of purified prothrombin. In addition, dilute hemophilic plasma was found to be equally as effective as dilute normal plasma in restoring the prothrombin time of stored plasma. It is evident from this that the plasma thromboplastin which we have described is not absent from hemophilic blood. Perhaps there are yet other substances in plasma that affect the rate with which prothrombin is converted to thrombin. On the other hand, there is the possibility that the plasma

TABLE 3. COMPARISON OF ACTIVITY OF DILUTE FRESH NORMAL PLASMA AND HEMOPHILIC PLASMA IN RESTORING THE 'PROTHROMBIN' TIME OF STORED PLASMA

	PROTHROMBIN TIME
	sec.
Stored human plasma.....	68
Stored plasma plus normal plasma (1:10 in saline) I.....	24
Stored plasma plus normal plasma (1:10 in saline) II.....	29
Stored plasma plus normal plasma (1:10 in saline) III.....	32
Stored plasma plus hemophilic plasma (1:10 in saline).....	27

thromboplastin of hemophilic blood, although present in normal amount, does not function normally due to the absence of an activator which makes it available for the clotting mechanism. This possibility is suggested by the work of Eagle and Harris (27), Mellanby and Pratt (28), Ferguson (29), Ferguson and Erickson (30), Tagnon, Davidson and Taylor (31), Quick (7) and Brinkhous (32).

The most recent work is that of Quick (7) and Brinkhous (32). Both have demonstrated the necessity for formed elements such as platelets in the normal clotting mechanism, and have discussed the significance of their findings with regard to the defect in hemophilia. According to both Quick and Brinkhous normal plasma, which is rendered platelet-free by centrifugation, clots very slowly on recalcification. Such plasma will not improve the coagulation time of hemophilic plasma which has also been rendered platelet-free, but the addition of platelets from either normal or hemophilic blood will bring about better coagulation of the mixture. However, this finding is interpreted differently by the two authors. Quick assumes that in the normal clotting mechanism, disintegrating platelets release an enzyme which converts inactive plasma thromboplastin to an active form which accelerates the conversion of prothrombin to thrombin in the presence of calcium. In hemo-



philia, according to Quick, the platelets are normal, but this inactive plasma thromboplastin is absent. According to Brinkhous the deficiency in hemophilia resides in a plasma factor required for platelet utilization.

#### SUMMARY

The rate of activation of purified prothrombin, in the presence of optimal calcium and rabbit brain emulsion, is accelerated to the same degree by the addition of small amounts of either normal or hemophilic sera. The 'prothrombin time' of stored plasma can be restored to the same degree by the addition of dilute plasma from either normal or hemophilic bloods. It is suggested that the blood plasma itself contains a substance which accelerates the activation of prothrombin, but that hemophilia is not the result of a deficiency in this particular factor.

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# VOLUME OF DISTRIBUTION OF POTASSIUM AND ITS ALTERATION BY SYMPATHOLYTIC AND ANTIHISTAMINIC DRUGS<sup>1</sup>

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THE suggestion has been made that the sympathetic nervous system controls, in a measure, the partition of potassium between the intracellular and extracellular fluid compartments. It is based on observations that stimulation of the sympathetic nervous system or administration of epinephrine alters the level of potassium in the blood and that administration of ergotamine to the rat lowers the volume of distribution of potassium as well as the concentration required to produce toxic cardiovascular effects (1). In the dog, volumes of distribution of potassium calculated before full equilibration show a similar reduction after administration of ergotamine, but no changes could be detected in the concentrations required to produce the several characteristic electrocardiographic indications of potassium toxicity or in the concentrations required to produce death (2). It was therefore thought possible that the reduction in volumes of distribution calculated in this manner reflected the peripheral vasoconstriction produced by ergotamine and not a direct effect of sympatholysis upon the ability of the body to distribute potassium.

It has in addition been observed that tetraethyl ammonium, a substance of quite dissimilar chemical nature and general pharmacological properties also possesses sympatholytic properties. It has quite the opposite effect, and protects the organism against the toxic effects of potassium, both in the dog and in the amphibian (3).

The various sympatholytic compounds have, however, properties apart from their sympatholytic effects, and these might well account for the diversity of results thus far obtained. A study was therefore undertaken of the influence of a variety of sympatholytic compounds on the volume of distribution of potassium in the dog, steps being taken to insure that opportunity was given for full equilibration before final determinations were made. As a further control, and because of reports indicating that antihistaminic agents alter cell or capillary permeability, a group of these compounds was also studied.

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## METHODS

Two groups of experiments were performed. In the first group, the toxicity of potassium was studied in 38 dogs. Animals were anesthetized with sodium barbital, 300 mg/kg. intravenously. An indwelling cannula was placed in the femoral vein and isotonic (1.12%) potassium chloride solution was infused at a slow drip until death occurred. Frequent electrocardiographic records were taken, using a Sanborn Visocardiette (lead II), by means of which the end-points of the various toxic effects could be adjudged and correlated with the level of serum potassium attained (4). Serum potassium was determined initially, after administration of the drug, and at various stages throughout the course of intoxication and the level of potassium determined in plasma samples by means of flame photometry (5). In addition to a control group of animals, potassium toxicity was determined in groups receiving *a*) N,N-dibenzyl- $\beta$ -chloroethylamine (Dibenamine), 20 mg/kg.; *b*) piperidylmethylbenzodioxane (933F), 5 mg/kg.; *c*) 2-benzyl-2-imidazoline (Priscol), 10 mg/kg.; *d*) pyridyl-benzyl-

TABLE 1.<sup>1</sup> AVERAGE VALUES AND STANDARD DEVIATIONS FOR EFFECTS OF VARIOUS DRUGS ON SERUM POTASSIUM LEVEL

DRUG	NO. OF DOGS	WEIGHT (KG.)		KCl, 1.12% (TOTAL CC.)		KCl, 1.12% (CC/KG.)		SERUM K (mg. %)							
								Initial		Post-drug		Loss of P		Terminal	
		Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$
Control.....	6	10.5	2.53					14.0	2.02			36.7	4.24	47.6	9.33
Priscol.....	6	7.5	2.13					14.0	0.95	15.4	1.29	33.8	3.07	45.8	9.07
Dibenamine.....	8	9.9	1.46	297	72.1	29.8	5.57	12.5	0.72	12.3	1.37	34.6	3.64	40.9	6.26
Pyribenzamine....	7	9.1	2.95	231	92.4	25.7	6.95	17.4	2.23	17.8	1.44	39.5	1.09	43.5	1.90
933F.....	3	6.2	0.55	85	38.9	13.2	5.02	16.5	1.16			39.6	0.0	44.5	2.27
Antistine.....	8	9.3	2.46	163	22.4	17.2	6.95	14.5	1.80			36.5	2.29	45.7	7.81

<sup>1</sup> The complete data for tables 1 and 2 have been deposited with the American Documentation Institute, 1719 N Street N. W., Washington 6, D. C. For copies of these order Document 2869 directly from the American Documentation Institute, remitting \$0.50 for microfilm images 1 inch high on 35 mm. film or \$0.50 for photo copies (6 x 8 inches).

dimethylethylene-diamine (Pyribenzamine), 3 mg/kg.; and *e*) 2-(N-benzylanilino-methyl-methyl) imidazoline (Antistine), 3 mg/kg.

In the second group of 32 dogs, animals were also anesthetized with sodium barbital, administered intravenously. Two dogs were paired in each experiment. The ureters were ligated some time before the experiment to produce anuria, a cannula was inserted into a femoral vein for administration of isotonic potassium chloride, and a Sanborn Visocardiette connected by lead II. Blood samples were obtained by jugular puncture for the determination of serum potassium by flame photometry (5).

After control blood samples and electrocardiograms were obtained, the injection was continued until disappearance of the P wave, this point being selected because it represents an advanced stage in the cardiac effects of potassium, but still permits some latitude in the administration of potassium before the development of intraventricular block and final cardiac arrest.

Upon disappearance of the P wave injection was stopped and 5 to 10 minutes permitted to elapse for equilibration to occur. If at the end of that time P waves had not returned, a period of one hour was allowed before the volume of distribution of potassium was determined. If however the P waves returned within the period of 5 to 10 minutes, the drip was restarted and maintained until the P waves again disappeared. Usually only a few cubic centimeters of infusion solution were necessary to accomplish this. Experience indicates that if potassium chloride has been administered slowly, then, when the P waves disappear, only a total of 20 to 40 cc. more of isotonic potassium chloride can be administered, and only over a period of several hours.

During the hour that was allowed for distribution, the P waves usually returned to a variable degree. After the volume of distribution had been determined at the end of the hour, one of the dogs received Dibenamine, 20 mg/kg., intravenously over a 5-minute period. The volume of distribution was again determined for both dogs after an interval of one-half and one hour after the Dibenamine was given. At the end of the hour, administration of potassium chloride was then restarted for both dogs, and continued until the P waves disappeared. The drip was then stopped,

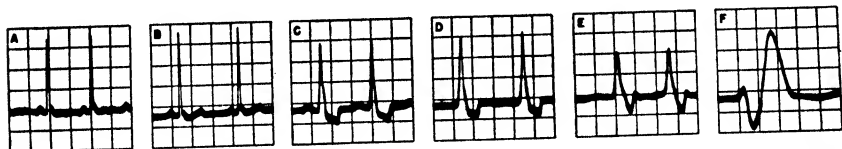


Fig. 1. VOLUME OF DISTRIBUTION of potassium and its alteration by sympatholytic and antihistaminic drugs. Dog 16-K. Dibenamine 20 mg/kg. intravenously between 1:23 and 1:27 p.m. A. ECG taken at 1:48 p.m. after return to normal state. B, C, D, E, F. Records showing successive stage of increase in amplitude of T wave, flattening and disappearance of P wave, and depression of S-T segment. Intraventricular block and arrest characteristic of potassium intoxication.

another hour allowed for equilibration, and the volume of distribution was again determined.

## RESULTS

Table 1 summarizes the data of the first group of experiments. It shows clearly that none of these agents significantly altered the normal resting level of serum potassium or influenced the general toxicity of potassium, as shown by the level at which the various characteristic electrocardiographic signs of potassium intoxication and death took place. In addition, the general sequence of events and the qualitative picture of the sequence of electrocardiographic changes during potassium intoxication remained the same, as illustrated in figure 1.

Certain electrocardiographic changes were, however, observed after administration of the substance under study, and these can be reviewed briefly as follows (fig. 2):

*Priscol* produced the most marked and consistent changes. There was a temporary increase in rate and in the amplitude of the P and T waves. The amplitude of R was reduced. *Dibenamine* had in general the same effects as *Priscol* in that the rate

was increased without change in P and R. T was increased in amplitude. 933F produced no outstanding deviation in the doses employed. *Pyribenzamine* caused a moderate slowing in the rate and a temporary increase in the amplitude of T. *Antisthine* produced a transient increase in heart rate and a temporary increase in the amplitude of the T wave.

The only consistent change produced by all drugs employed was a transient increase in the amplitude of the T wave which was, as can be seen from table 1, not associated with the alteration in the serum potassium.

During the administration of potassium to animals which had received Dibenamine, it was observed that considerably larger quantities of potassium were required to produce a given toxic level than seemed necessary in the other animals and from past experience, and this general impression was confirmed when the lethal quantities of potassium were calculated in cubic centimeters of isotonic potassium chloride per kg. of body weight. Since the level at which death occurs had not been altered by

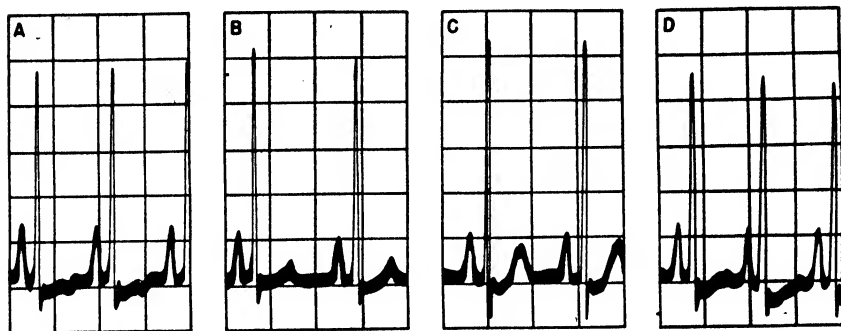


Fig. 2. VOLUME OF DISTRIBUTION of potassium and its alteration by sympatholytic and antihistaminic drugs. Dog 7-K. A. Control ECG. B. ECG taken 2 minutes after *Pyribenzamine* 3 mg/kg. intravenously, showing beginning elevation of T wave and slight increase in amplitude of R. C. ECG taken 3 minutes after injection of *Pyribenzamine*. Maximum R and T changes. D. Virtual return to normal 10 minutes after injection.

administration of Dibenamine, it seemed clear therefore that this drug must have increased the rate at which potassium was able to leave the blood stream or the total quantity leaving the extracellular fluid. In the latter event, the volume of distribution of potassium must have been increased. It was this observation that led to the detailed studies of the volume of distribution of potassium in the next section.

Table 2 presents the complete data of the second group of experiments, and indicates the salient results which can be summarized briefly:

a) In the control animals an average volume of distribution of 97.9 per cent of body weight was achieved at 60 minutes after cessation of injection; by 90 minutes it had increased to 108 per cent; and by 120 minutes to 114 per cent. The average of all determinations was 103 per cent.

b) In Dibenamine-treated animals an average volume of distribution of potassium of 120 per cent was reached in 60 minutes, 135 per cent in 90 minutes, and 143 per cent in 120 minutes. The average of all determinations was 132 per cent.

## DISCUSSION

These observations dispose at once of any possibility that sympatholysis per se lowers the volume of distribution of potassium. The lowered volume of distribution of potassium noted after ergotamine must therefore be related to some side-effect of the drug, probably, as has been suggested, its peripheral vasoconstrictor effect. This suggests caution in attributing the increased volumes of distribution noted after Dibenamine to the sympatholytic effect until more complete data are available. It seems, however, that circulatory factors, governing simply rate of transfer of potassium, cannot be solely responsible. As can be seen in the controls, average volumes of distribution were 108 per cent at 90 minutes and 114 at 120 minutes, suggesting that distribution was leveling off and would not have been significantly higher even if more time were allowed for equilibration, as would be expected if the higher values after Dibenamine resulted solely from the factor of rate of distribution.

The second point of interest in the data presented here is the high values found for the volume of distribution of potassium in normal animals. The only other de-

TABLE 2. AVERAGE VALUES AND STANDARD DEVIATIONS FOR VOLUME DISTRIBUTION OF POTASSIUM FOR A SERIES OF CONTROL AND DIBENAMINE-TREATED DOGS

DRUG	NO. OF DOGS	WEIGHT (KG.)		KCl, 1.12% (TOTAL CC.)		KCl, 1.12% (cc/KG.)		INITIAL K LEVEL (MG. %)		VOLUME DISTRIBUTION (%)									
										60 MINUTES		90 MINUTES		120 MINUTES		TOTAL			
		Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$	Av.	$\sigma$	No. det.	Av.	$\sigma$	No. det.	Av.	$\sigma$	No. det.	Av.	$\sigma$	
Control	28 <sup>1</sup>	0.7	6.11	27.2	05.4	27.6	7.68	15.7	1.09	31	97.9	18.5	0	108	20.8	49	103	21.5	
Dibenamine	20	0.6	1.07	32.2	102.8	32.6	8.42	16.2	1.09	20	120.	29.7	14	135	22.8	59 <sup>2</sup>	132	37.1	
Standard error of difference between two means										7.43		9.24		13.6		5.72			

<sup>1</sup> Control determinations was made on dogs before giving Dibenamine. A total of 32 dogs was used.

<sup>2</sup> This figure includes 5 dogs pretreated with Dibenamine.

terminations that have been reported are those of Winkler and Smith who found values averaging 75 per cent. The coincidence of this figure with that for total body water permitted the implication that injected potassium was distributed more or less uniformly in the body fluids. The accumulation of potassium in high concentration within cells during growth and the apparent affinity of the liver for potassium in excess of serum concentrations (6) make it more reasonable to suppose the injected potassium is not simply diffused through the intracellular and extracellular fluid spaces, but is accumulated in some regions in concentrations in excess of that in the serum. The high volumes of distribution found here require some such unequal distribution of potassium. Where and how this is achieved cannot be suggested from the present experiments.

What regulates the maximum value of the volume of distribution is equally unknown. Clearly, in the growing animal, potassium has an extremely high volume of distribution, since for each milliequivalent added to a liter of extracellular fluid, 40 or 50 are added to the adjoining 2 liters of intracellular space. Concentration of added

potassium within the cells in anything resembling the normal partition between cells and serum cannot therefore be envisaged here. Pointing in the same direction is the inability of the nephrectomized anuric animal to restore to intracellular spaces the potassium released in the daily metabolism, for within a few days the release of endogenous potassium into the blood stream increases the concentration of serum potassium to a lethal level.

#### SUMMARY

A variety of sympatholytic and antihistaminic agents were investigated with regard to their influence on the toxicity of injected potassium and its distribution within the body. None of them altered significantly the normal resting level of serum potassium or influenced the level at which the various characteristic electrocardiographic signs of potassium intoxication and death took place.

In control animals the average volume of distribution determined after 60, 90, and 120 minutes of equilibration was 103 per cent of the body weight. Animals treated with Dibenamine showed comparable values averaging 132 per cent.

These observations indicate that in the normal animal added potassium must be stored in some body compartments in concentrations higher than in plasma and that this process is facilitated by Dibenamine. There is no evidence that this represents a result of the sympatholytic effect of Dibenamine.

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# URINE FLOW AND SOLUTE EXCRETION OF HYDROGENIC DOG UNDER 'RESTING' CONDITIONS AND DURING OSMOTIC DIURESIS

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PREVIOUS papers (1-4) have dealt with the osmotic limitations of the kidney of hydropenic man under conditions of solute loading. The relation of solute excretion to urine flow and changes in renal osmotic work during osmotic diuresis were described. The present paper deals with the solute excretion and urine flow of the normal dog kidney studied under similar conditions.

During osmotic diuresis produced by a wide variety of substances in the hydropenic subject, a constant relation between urine flow and the rate of solute excretion or the urinary load was shown to exist. Urine flow under these conditions was independent of the chemical or ionic properties of the urinary solutes and dependent only on their quantity (load) in the urine. The plasma level, the filtration rate and the mode of excretion of the urinary solutes determined urine flow only insofar as they modified the magnitude of the load. The identity and reproducibility of the relation of flow to load, regardless of wide variations in urinary composition, led to the assumption that distal to a spatially distinct point in the renal tubule, the percentage composition of the urine solutes remains constant and only water is reabsorbed.

Data from 171 urine periods collected during 23 experiments on 7 female and 2 male mongrel dogs are presented. Three of the animals were studied in multiple experiments; single experiments were performed on the others. The diet consisted of commercial dog food, horse meat and dog biscuits and was supplemented with ferrous sulfate.

To insure a state of hyponatremia, the dogs were deprived of water for 20 hours and fed a meal of moderate to high-protein content 16 hours before experimentation. The influence of diet on the experimental results is discussed later.

The experimental work presented here was undertaken with several goals in mind: first, to aid in evaluating the conclusions derived from the study in man; secondly, to provide a base line for studies of the osmotic limitations during experimentally induced renal insufficiency; and finally, by the use of electrolytes as loading solutes, to gain some insight as to the renal mechanisms controlling electrolyte excretion. The previous study in man and preliminary experiments in the dog showed, after loading with certain electrolytes, deviations from the characteristically reproducible pattern of ion excretion produced by non-electrolytes. The following solutes were used: mannitol, sodium chloride, sodium thiocyanate, sodium nitrate, sodium bicarbonate, sodium para-aminohippurate, sodium phosphate, sodium sulfate, sodium

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thiosulfate and sodium ferrocyanide. The present paper deals with the urine flow and solute excretion of the kidney in the 'resting' hydropenic state and during osmotic diuresis. Other papers will deal with the specific interionic effects observed and the osmotic work.

#### EXPERIMENTAL

Most of the experiments were performed without anesthesia, the animals having been trained to lie quietly with loose restraint during the procedure. Nembutal anesthesia, which does not appear to affect the osmotic limitations of the kidney during solute loading, was used for the experiments in which large loads of sodium chloride, sodium ferrocyanide and sodium thiocyanate were administered. Urine was collected in a syringe attached to an indwelling catheter. In the bicarbonate experiments and in 10 experiments with other electrolytes, the urine was placed under oil for the determination of carbon dioxide content and pH. Blood was obtained by puncture of the femoral artery or, more frequently, by venous catheterization or venipuncture. It was placed under oil in the bicarbonate and sodium chloride experiments, and in heparin tubes in the other experiments.

The easily soluble loading substances were administered in a concentration of 3800 milliosmols/l. Mannitol, sodium bicarbonate and sodium ferrocyanide were given in saturated solution. The sodium para-aminohippurate solution contained 1840 milliosmols per liter. For the calculation of the osmolar weights of the electrolytes, the molecular weight was divided by the number of ions constituting the molecule. The sodium phosphate solution was made to a pH of 7.3 by mixing dibasic and monobasic phosphate in a ratio of 77 to 23.

The general procedure of the experiments was as follows: after collecting one or more preliminary urine and blood specimens, the loading solute was given intravenously in a rapid injection. The priming dose, calculated to impose approximately the same load in each experiment, amounted to 30 milliosmols per liter volume distribution of the solute. This would amount, for example, in the case of a pentenary electrolyte such as  $\text{Na}_4\text{Fe}(\text{CN})_6$ , to 6 mm of the salt per liter volume distribution of ferrocyanide. Such a dose would raise the ferrocyanide level to 6 milliosmols and the sodium level by 24 milliosmols/l. of plasma. A volume of distribution of 20 per cent of the body weight was assumed for mannitol, sulfate, ferrocyanide, phosphate and para-aminohippurate, one of 25 per cent for nitrate and bicarbonate, of 27 per cent for thiocyanate and of 30 per cent for thiosulfate. Changes in the volume of distribution produced by shifts of fluid from the intracellular space as a result of the injection of a hypertonic solution were neglected.

After the priming injection, the plasma level of the solute was maintained by continuous infusion at a rate approximately equal to its disappearance rate by means of a constant infusion machine. The disappearance rates, expressed as per cent of the amount of the injected solute disappearing per minute, used in the calculation of the maintenance infusions were 0.5 per cent for nitrate and thiocyanate; one per cent for mannitol, thiosulfate, sulfate and ferrocyanide; 1.5 per cent for phosphate and bicarbonate; 2.5 per cent for para-aminohippurate. A period of 15 to 20 minutes was allowed for equilibration of the priming dose. Urine and blood were then collected at appropriate intervals, usually 10 to 20 minutes, for 3 periods. A second priming dose, equal in amount to the first, was then injected and 15 to 20 minutes allowed for its equilibration. The new plasma level was maintained by continuous infusion at double the rate of the first maintenance and 3 more urine and blood specimens were collected.

The procedure for certain experiments was modified. The dose of sodium chloride was calculated to raise the osmolarity of all body fluids by 100 milliosmols/l. and no maintenance infusion was given. In one experiment with ferrocyanide the priming infusions were calculated to add 60 milliosmols, and in the para-aminohippurate and thiocyanate experiments, 20 milliosmols/l. of distribution volume. A single priming infusion was given in one experiment with para-aminohippurate and in one with thiocyanate. For measurement of filtration rate and renal blood flow during 3 mannitol experiments, saturated solutions of creatinine and para-aminohippurate were given with the priming and maintenance infusions of mannitol.

The analytical methods for urea, sodium, chloride, potassium, phosphate, sulfate, para-aminohippurate and mannitol have been described previously (2). Thiocyanate was determined by colori-

metric measurement of ferric thiocyanate, nitrate by a modification of the method of Noll (5), bicarbonate according to Van Slyke (6), thiosulfate by the method of Gilman *et al.* (7) and ferrocyanide by that of Van Slyke *et al.* (8). The urine pH was determined approximately with indicators. Interference of ferrocyanide in the determination of chloride was circumvented by precipitation with  $ZnSO_4$  prior to titration. In the determination of chloride on specimens containing thiocyanate, the contribution of thiocyanate was subtracted from the total halide value. To correct for the small error in the  $CO_2$  determination due to sulfur dioxide formation in urine containing thiosulfate, a value based on the amount of gas formed from a solution of thiosulfate of equal strength was subtracted.

Osmolarity of urine was determined by the freezing point depression. In the bicarbonate experiments, the difference in  $CO_2$  content before and after freezing was added to the osmolarity determined from the freezing point depression.

In general, the animals suffered few adverse effects from administration of the solutes. Retching and vomiting occurred occasionally during priming with all solutes except sodium para-aminohippurate and sodium bicarbonate. The absence of discomfort after para-aminohippurate was surprising in view of the symptoms produced in man at much lower levels. Sodium bicarbonate had no noticeable effect—not even on ventilation. After the injection of a large dose of sodium chloride, marked overventilation of about 15 minutes duration occurred. At the termination of all experiments, except

TABLE 1. URINE FLOW AND SOLUTE CONCENTRATIONS IN URINE AND PLASMA UNDER 'RESTING' HYDROGENIC CONDITIONS

	FLOW	UREA	Na	K	Cl	$SO_4$	$PO_4$	SUM SOLUTES DETER.	TOTAL OSMO- LARITY CALC. <sup>1</sup>
	cc/min/ m. <sup>2</sup>								
Urine.....	.25 ±.01	1135.6 ±64.4	83.1 ±8.0	68.2 ±5.7	86.0 ±8.6	53.9 ±2.2	34.3 ±5.2	1392.2 ±63.0	1871.3 ±77.8
Plasma.....		9.9 ±0.5	155.2 ±1.4	4.2 ±0.1	120.6 ±1.1	2.0	1.4	286.4 ±8.3	319.7 ±3.0

Means and standard errors of 27 periods on 7 dogs in 18 experiments. Concentrations are expressed in milliosmols per liter.

<sup>1</sup> The principles of calculating total osmolarity of urine (2, footnote 2) and plasma (4, table 1, footnote 1) have been described previously.

the one with phosphate, the dogs were in excellent condition. They were thirsty and drank large quantities of water.

The only immediate effect of thiocyanate injection on the unanesthetized animal was to produce vomiting. Shortly after the experiment, however, marked ataxia and lethargy developed and persisted for 2 days. The dog eventually recovered. Following the infusion of phosphate, sustained tetany, involving the entire body, ensued. The animal seemed out of contact with its environment and somewhat depressed. At the termination of the experiment, the tetany subsided somewhat, and the dog was, for a time, more cognizant of its surroundings but refused to drink. The body temperature at that time was 42.8° C. Overventilation developed and continued until death, 5 hours after termination of the experiment. Autopsy revealed only marked dehydration. The hyperthermia may be attributed to increased heat production caused by tetany combined with inadequate heat dissipation and the dehydration to osmotic diuresis accompanied by overventilation. The data of the last 3 periods of this experiment are omitted from the presentation because of the complicating factors described.

## RESULTS

*Solute Excretion of the 'Resting' Hydropenic Kidney.* In table 1 are presented the mean values with their standard errors of the concentration of solutes in urine and

plasma during 27 preliminary periods studied in 18 experiments. A comparison of the urinary values with those obtained in a similar study in man (4) shows considerable differences. The calculated total osmolarity of urine in the dog was on the average 50 per cent higher and showed greater variation, the values for individual specimens differing as much as 2-fold. Specimens with osmolarities as low as 1200 and as high as 2800 milliosmols/l. were obtained. The urine flow, in contrast to the osmolarity, showed the same variation and was of the same order of magnitude as in man. Among the individual urinary solutes, urea constituted a relatively constant proportion of the total, about 60 per cent. An average of 25 per cent of the solutes was not determined by analysis. The electrolyte content of the urine, amounting to

about 15 per cent of the total solutes, was both proportionately and absolutely less than in man.

Plasma osmolarity was about 5 per cent greater than in man. The greater total osmolarity was accounted for by higher concentrations of sodium, chloride and urea. The values were similar to those previously reported by Dill *et al.* (9).

In the course of the study it became apparent that the main factor determining the osmolarity of preliminary specimens was the type of meal given 16 hours prior to experimentation. The urine after a meal containing a large amount of horse meat

had a significantly greater osmolarity than that after a meal of moderate or low meat content. As mentioned, the percentage composition was the same for urines of high and low osmolarity, with urea accounting for a relatively constant percentage of the total solutes. Meat feeding had no effect on the rate of urine flow. In figure 1 is presented a scatter graph showing the osmolarity of the preliminary urines plotted against urine flow. No definite pattern is discernible.

The absence of a relation between urine flow and osmolarity under 'resting' conditions, in sharp contrast to the well-defined relation observed after loading, is of great interest and raises several questions. The data suggest that during hydropenia a minimum of urine flow may exist, independent of wide variations in urine osmolarity. The renal mechanisms effecting such a phenomenon are difficult to visualize. If it is assumed that the tonicity of the urine in the proximal tubule is similar to that of plasma and that urine flow is constant at a minimal rate, the volume of urine

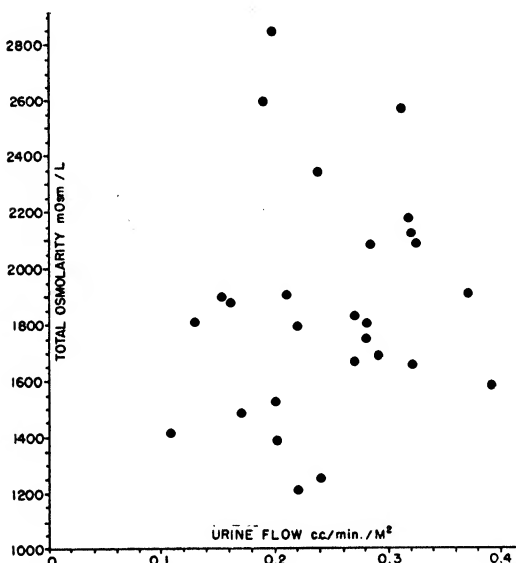


FIG. 1. URINE OSMOLARITY VERSUS FLOW under 'resting' hydropenic conditions. Data for 27 periods.

entering the distal tubule will of necessity be greater the higher the osmolarity of the final urine. To achieve a constant rate of urine flow, wide yet delicate adjustments in the rate of water reabsorption in the distal tubule will be required. To account for these adjustments, hitherto unrecognized forces governing water reabsorption must be hypothesized. Meat feeding, which has been shown to increase the urea clearance in both dog (10, 11) and man (12, 13), may effect some measure of adjustment in water reabsorption through changes in renal activity. For a complete understanding of the basic mechanisms involved, further study of the phenomenon will be required.

There is evidence that in man, also, differences in the diet may produce changes in the urine osmolarity during hydropenia similar to those in the dog. McCance (14) has shown that, in a subject deprived of water, a normal diet after one low in salt increased urine osmolarity from 1180 to 1430 milliosmols/l. and urine volume about 2-fold. Although the changes are of smaller magnitude than those observed in the dog, it is possible that the same basic mechanism is operative.

*Solute Excretion During Osmotic Diuresis.* In table 2 are shown the pertinent data on representative experiments with each of the 10 loading solutes used in this study. To conserve space, data for urine and plasma collected prior to loading and during equilibration of the priming injections have been omitted. The table contains information on the weight and surface area of the dogs and the volume and concentration of the infusions. The tabulated data include the urine volume and the concentrations of the loading solutes as well as of the major constituents of urine and plasma. For 5 of the experiments the urine pH is listed.

*Urine Flow and Total Solute Excretion.* Urine flow increased from an average pre-loading value of 0.25 cc/minute to as much as 14 cc/minute after mannitol loading. The higher rates of flow obtained in this study, equivalent to about 20 per cent of the filtration rate, are comparable to those obtained in similar experiments in man. Considerable differences in the diuretic efficiency of the various solutes are apparent. Urine volume was only slightly increased by thiocyanate while mannitol loading produced a copious diuresis. The factors determining the diuretic efficiency of a solute have been discussed in detail elsewhere (2).

For the osmolarity of the urine, 2 values have been listed, 1) the calculated total osmolarity, indicating the actual number of solute particles in the urine, derived from the freezing point depression and the concentrations of individual solutes by a method described previously and 2) the effective osmotic activity calculated by the formula,

$$\text{Effective Osmotic Activity} = \frac{\Delta}{1.86} \times 1000$$

in which  $\Delta$  is the observed freezing point depression and 1.86 is the molecular lowering of the freezing point of a solution of an ideal solute in water. In the case of electrolytes, two deviations from ideality have to be considered: 1) dissociation into ions, which increases the number of osmotically active particles and 2) inter-ionic attraction which diminishes their osmotic activity. The activity of an electrolyte solution is given by its osmotic pressure. The deviation from the ideal is measured by the

TABLE 2. URINE FLOW AND SOLUTE CONCENTRATIONS IN URINE AND PLASMA DURING OSMOTIC DIURESIS

PERIOD, SPECI- MEN	CONCUR- RENT TIME	URINE VOL.	MANNI- TOL	UREA	Na	K	Cl	PO <sub>4</sub>	SO <sub>4</sub>	CO <sub>2</sub>	pH	SUM SOL- UTES DE- TER. <sup>1</sup>	TOTAL OSMO- LAR- ITY CALC.	EF- FEC- TIVE OSMO- TIC ACTIVITY	
		min.	cc/min/ m. <sup>2</sup>												
I. Mannitol. Dog De., 16.6 kg.; 0.64 m. <sup>2</sup>															
a. 0 to 10 min.—106 cc. 25% mannitol. Maintenance 0.01 cc/min.															
1, U	14-28	8.63	320.3	46.8	56.5	13.3	54.4					490	569	552	
P	21		38.1	8.1	153.6	4.11	113.2					318	354		
2, U	28-47	6.32	340.6	57.5	56.5	14.8	52.8					533	636	617	
P	34		34.8	7.7	146.4	4.3	110.4					304	336		
3, U	47-64	6.69	371.6	61.0	55.5	18.4	52.4					572	665	640	
P	54		33.5	7.6	141.6	4.4	110.0					298	326		
b. 69 to 79-106 cc. 25% Mannitol. Maintenance 1.90 cc/min.															
4, U	85-102	12.3	329.4	30.4	44.5	16.2	48.6					475	551	536	
P	93		63.8	7.6	138.0	4.6	106.8					322	349		
5, U	102-121	12.5	344.5	32.5	41.0	15.0	46.6					487	566	552	
P	112		63.8	7.2	138.0	4.8	108.0					322	349		
6, U	121-137	11.9	357.6	33.6	37.0	18.5	43.6					498	579	565	
P	129		65.8	7.3	135.6	4.9	109.6					324	347		
II. NaCNS. Dog Is., 12.3 kg.; 0.56 m. <sup>2</sup>															
a. 0 to 7-15.4% NaCNS. Maintenance 0.033 cc/min.															
			CNS												
1, U	20-40	0.68	4.3	975.5	525	33.0	294.5	103.0	62.9	4.3		2003	2264	2090	
P	31		11.0	9.2	172.5	4.5	116.8	1.67				316	365		
2, U	40-60	0.81	5.0	750.9	545	48.0	357.7	83.2	52.8	5.8		1849	2004	1850	
P	50		11.7	9.2	175.5	4.7	116.8		.8			310	362		
3, U	60-80	0.67	6.4	615.5	530	40.0	366.8	68.7	45.5	3.5		1676	1863	1720	
P	70		12.3	8.6	174.0	4.7	116.4	1.5				318	358		
b. 82 to 89-15.4% NaCNS. Maintenance 0.166 cc/min.															
4, U	100-115	1.79	17.5	328.1	399	33.9	308.9	35.5		3.0		1126	1207	1135	
P	108		22.7	8.1	181.5	5.0	112.4	1.6				331	373		
5, U	115-130	1.58	17.3	274.1	369	24.0	296.0	31.6	10.3	2.8		1025	1135	1067	
P	123		22.8	8.4	181.5	5.1	110.0		.6			328	374		
6, U	130-145	1.54	19.0	312.0	384	28.5	313.4	32.9	13.4	2.7		1106	1154	1085	
P	137		24.1	8.0	177.0	5.3	110.0	1.5				326	365		
III. Na NO <sub>2</sub> Dog Pe., 18.4 kg.; 0.66 m. <sup>2</sup>															
a. 0 to 5 min.—37.5 cc. 16% NaNO <sub>2</sub> . Maintenance 0.21 cc/min.															
			NO <sub>2</sub>												
2, U	51-72	2.04	195.5	155.8	262.5	33.2	101.6				22.2	7.1	771	836	753
P	64		15.6	7.2	162.0	3.5	115.6						304	330	
3, U	72-97	1.92	193.7	153.7	295.0	38.8	95.6				24.1	7.1	701	834	750
b. 103 to 108 min.—37.5 cc. 16% NaNO <sub>2</sub> . Maintenance 0.42 cc/min.															
4, U	124-146	6.30	191.8	60.7	25.8	88.8					11.9	7.1	379	677	609
P	135		31.4	6.4	170.4	4.0	112.4						325	347	
5, U	146-158	6.52	220.8	61.0	265.0	22.5	87.2				11.7	7.2	677	693	624
P	151		32.9	6.3	172.8	4.0	111.6						328	352	
6, U	158-170	5.56	225.5	62.9	275.0	21.0	85.6				11.2	7.1	681	717	645
P	164		32.3	6.4	177.6	3.7	112.4						332	361	

TABLE 2—Continued

PERIOD, SPECI- MEN	CONCUR- RENT TIME	URINE VOL.	MANNI- TOL	UREA	Na	K <sup>+</sup>	Cl	PO <sub>4</sub>	SO <sub>4</sub>	CO <sub>2</sub>	pH	SUM SOL- UTES DE- TER. <sup>1</sup>	TOTAL OSMO- LAR- ITY CALC.	EF- FEC- TIVE OSMO- TIC AC- TIVITY
<i>min. cc/min/ m.<sup>2</sup></i>														
IV. NaCl. Dog Ra., 22.6 kg.; 0.68 m. <sup>2</sup>														
<i>a. 0 to 9 min.—89 cc. 2.7% NaCl.</i>														
2, U	26-50	8.01		48.3	295.0	22.8	274.0			2.7		643	718	645
P	47			7.2	189.6	4.8	163.0					383	388	
3, U	50-72	4.60		64.4	337.5	25.8	306.0			2.7		736	815	734
P	70				189.6	4.6	162.2					376	388	
4, U	72-107	3.38		91.0	327.5	37.3	313.4			2.7		772	870	786
P	105			7.1	188.4	4.8	161.2					381	386	
5, U	108-157	2.13		120.0	337.5	48.3	320.6			3.3		830	912	827
P <sub>1</sub>	147			5.5	181.2	4.2						210	368	
P <sub>2</sub>	154			5.6	182.4	4.2	156.4					368	371	
6, U	157-196	1.26		185.5	345.0	61.5	354.6			5.8		952	1082	985
P	192			5.6	180.0	4.4	155.4					366	366	
V. Na HCO <sub>3</sub> . Dog Pe., 17.5 kg.; 0.65 m. <sup>2</sup>														
<i>a. 0 to 10 min.—69 cc. 8% NaHCO<sub>3</sub>. Maintenance 1.03 cc/min.</i>														
1, U	20-32	2.65		202.6	307.5	42.8	48.8			238.4	8.6	840	896	848
P	26			6.4	160.0	3.8	110.0			35.8	7.58	318	328	
2, U	32-44	2.43		207.8	322.5	38.7	36.4			270.8	8.6	876	943	888
P	38			8.4	160.5	3.8	110.0			34.8	7.60	318	329	
3, U	44-56	2.43		215.5	345.0	41.0	31.6			287.6	8.6	921	964	904
P	50			7.7	159.0	3.8	108.0			36.7	7.64	316	325	
<i>b. 58 to 68 min.—69 cc. 8% NaHCO<sub>3</sub>. Maintenance 2.03 cc/min.</i>														
4, U	78-90	6.94		74.6	303.0	17.9	35.6			242.2	8.6	673	677	635
P	84			7.4	165.0	3.5	106.4			46.4	7.69	329	336	
5, U	90-102	5.80		88.2	307.5	30.9	20.0			281.7	8.6	728	740	688
P	96			7.0	170.0	3.4	104.4			48.2	7.60	333	346	
6, U	102-114	6.59		77.8	310.5	28.5	27.2			277.1	8.6	721	744	692
P	108			7.8	173.0	3.2	105.6			47.4	7.69	337	352	
VI. Na p-aminohippurate. Dog Be., 11.4 kg.; 0.55 m. <sup>2</sup>														
<i>a. 0 to 5 min.—30 cc. 20% NaPAH. Maintenance 0.75 cc/min.</i>														
			PAH											
1, U	20-32	2.36	278.0	234.5	237.0	45.0	4.4	14.9				815	912	842
P	25		10.1	11.0	162.0	3.6	122.4	1.5				311	334	
2, U	32-44	2.70	289.3	210.9	264.0	45.9	6.8	16.2				835	909	835
P	38		10.7	11.2	162.0	3.7	126.4	1.5				316	335	
3, U	44-56	2.97	291.5	201.7	289.5	41.0	12.0	18.7				857	926	848
P	50		11.8	10.8	170.0	3.7	126.0	1.5				324	350	
<i>b. 58 to 64 min.—30 cc. 20% NaPAH. Maintenance 1.50 cc/min.</i>														
4, U	70-91	6.08	268.9	99.5	274.5	25.8	14.2	9.7				693	725	656
P	85		21.0	10.0	176.0	3.5	121.6	1.5				334	361	
5, U	91-103	6.30	259.8	91.3	285.0	23.2	16.4	10.3				686	714	644
P	97		22.6	9.8	174.0	3.5	122.4	1.6				334	357	
6, U	103-115	6.88	252.4	82.6	244.5	19.0	20.8	10.0				638	691	624
P	109		23.6	9.9	179.0	3.7	120.4	1.6				338	367	

TABLE 2—Continued

PERIOD, SPECI- MEN	CONCUR- RENT TIME	URINE VOL.	MANNI- TOL	UREA	Na	K	Cl	PO <sub>4</sub>	SO <sub>4</sub>	CO <sub>2</sub>	pH	SUM SOL- UTES DE- TER. <sup>1</sup>	TOTAL OSMO- LAR- ITY CALC.	EF- FEC- TIVE OSMO- TIC AC- TIVITY
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min. cc/min/  
m.<sup>2</sup>

VII.  $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ . Dog Ja., 17.4 kg.; 0.65 m.<sup>2</sup>

a. 0 to 6 min.—25.5 cc. 15%  $\text{Na}_2\text{HPO}_4$ ; 3.8%  $\text{NaH}_2\text{PO}_4$ . Maintenance 0.37 cc/min.

1, U	16-29	3.34		187.9	230.0	108.0	9.2	238.7		2.5	6.4	776	796	681
P	20			10.1	171.6	4.7	124.8	11.9				323	345	
2, U	29-44	2.94		149.0	337.5	71.0	4.0	258.1		4.5	6.8	824	894	733
P	36			10.4	172.8	3.9	124.0	11.5				323	347	
3, U	44-57	3.48		135.6	360.0	60.0	8.4	232.2		7.4	6.8	804	830	687
P	50			10.5	172.8	3.8	126.8	11.3				325	347	

b. 60 to 70 min.—25.5 cc. 15%  $\text{Na}_2\text{HPO}_4$ ; 3.8%  $\text{NaH}_2\text{PO}_4$ . Maintenance 0.76 cc/min.

4, U	75-88	5.65		63.8	290.0	51.0	7.8	193.5		9.3	7.2	615	669	537
P	80			10.2	189.6	4.1	124.0	20.3				348	373	
5, U	88-100	4.85		64.8	285.0	52.5	4.4	212.9		7.3	7.2	627	663	522
P	93			10.2	189.6	4.7	125.2	21.9				352	373	
6, U	100-112	3.36		64.9	280.0	66.5	3.2	232.2		5.7	6.9	652	730	580
P	107			9.7	190.8	4.8	123.6	23.4				352	374	

VIII.  $\text{Na}_2\text{SO}_4$ . Dog Pe., 18.6 kg.; 0.67 m.<sup>2</sup>

a. 0 to 4 min.—30 cc. 18%  $\text{Na}_2\text{SO}_4$ . Maintenance 0.30 cc/min.

1, U	20-40	5.62		107.6	367.5	25.4	12.8	155.5				669	718	591
P	30			11.6	186.0	3.6	117.2	14.7				333	368	
2, U	40-59	5.12		110.9	367.5	27.5	10.8	157.8				674	755	624
P	47							13.9						
3, U	59-86	4.70		122.1	417.5	35.0	6.8	176.4				758	854	692
P	72			10.1	192.0	3.5	119.2	13.4				338	380	

b. 91 to 98 min.—30 cc. 18%  $\text{Na}_2\text{SO}_4$ . Maintenance 0.60 cc/min.

4, U	114-123	7.26		65.7	437.5	21.9	6.4	174.4				706	761	618
P	136				212.4	3.4	123.2	24.8				364	409	

IX.  $\text{Na}_2\text{S}_2\text{O}_8$ . Dog Pe., 17.8 kg.; 0.66 m.<sup>2</sup>

a. 0 to 6 min.—42 cc. 20%  $\text{Na}_2\text{S}_2\text{O}_8$ . Maintenance 0.42 cc/min.

1, U	17-33	4.42	SO <sub>4</sub>	180.5	117.6	340.0	25.3	7.4		26.3	3.2	6.4	700	791	635
P	25			9.7	10.2	182.4	3.8	121.2					327	365	
2, U	33-46	4.16		191.0	129.2	382.5	25.8	7.0		29.6	3.6	6.5	769	842	674
P	40			9.7	10.3	177.6	3.8	121.6					323	355	
3, U	46-58	4.07		196.5	130.8	395.0	25.8	2.2		31.0	4.2	6.7	786	868	696
P	52			9.7	9.7	176.4	3.3	124.3					324	351	

b. 61 to 67 min.—42 cc. 20%  $\text{Na}_2\text{S}_2\text{O}_8$ . Maintenance 0.84 cc/min.

4, U	75-91			184.0	68.9	402.5	15.0	3.6		19.7	9.8	7.0	704		
P	84			20.0	9.1	193.2	3.3	122.4					348	374	
5, U	91-101	7.96		188.5	66.8	407.5	15.5	0.8		21.2	9.4	7.1	710	770	610
P	96			19.7	9.4	193.2	3.2	122.4					348	374	
6, U	101-113	8.40		190.5	67.8	402.5	15.3	1.0		21.7	10.2	7.0	709	779	617
P	107			20.0	8.6	199.2	3.0	123.6					354	385	

TABLE 2—Continued

PERIOD, SPECI- MEN	CONCUR- RENT TIME	URINE VOL.	MANN - TOL	UREA	Na	K	Cl	PO <sub>4</sub>	SO <sub>4</sub>	CO <sub>2</sub>	pH	SUM SOL- UTES DE- TER. <sup>1</sup>	TOTAL OSMO- LAR- ITY CALC.	EF- FEC- TIVE OSMO- TIC AC- TIVITY
min.		cc/min/ m. <sup>2</sup>												
X. NaFe(CN) <sub>6</sub> . Dog De., 15.9 kg.; 0.64 m. <sup>2</sup>														
a. 0 to 5 min.—25 cc. 19% NaFe(CN) <sub>6</sub> . Maintenance 0.25 cc/min.														
			Fe- (CN) <sub>6</sub>											
1, U	17-29	1.72	167.1	216.1	542.5	71.0	12.0							
P	23		5.2	8.4	174.0	4.1	116.0			2.2	6.0	1011	1155	755
												308	341	
2, U	29-44	1.95	183.8	220.4	600	82.5	12.8							
P	36		4.6	8.8	172.8	4.1	116.0			2.4	6.4	1102	1245	814
												306	341	
3, U	44-59	1.67	183.0	224.2	615.0	108.0	15.8							
P	51		4.5	8.4	171.6	4.1	118.5			10.3	6.7	1156	1290	855
												307	338	
b. 61 to 67 min.—25 cc. 19% NaFe(CN) <sub>6</sub> . Maintenance 0.50 cc/min.														
4, U	79-90	4.34	158.3	104.6	607.5	50.0	11.2							
P	84		8.6	7.8	186.0	4.0	115.5			20.1	7.0	952	1051	664
												322	354	
5, U	90-102	4.01	160.1	109.5	617.5	47.8	12.6							
P	96		8.2	8.0	184.8	4.0	114.0			25.7	7.1	973	1071	680
												319	353	
6, U	102-114	4.04	154.2	103.6	615.0	46.8	15.0							
P	108		7.9	7.4	186.0	3.9	115.5			30.8	7.2	965	1059	680
												321	356	

<sup>1</sup> The value for urine includes para-aminohippurate and creatinine. The value for plasma includes total CO<sub>2</sub>.

Plasma and urine concentrations are expressed as milliosmols per liter.

The concentrations of injection solutions are expressed as gm. of anhydrous salt/100 cc.

For the calculation of the total osmolality of plasma after loading with the multivalent electrolytes, the value (valence -1) X concentration multivalent anion was subtracted from the expression described previously (8, table 1, footnote 1).

ratio of the actual osmotic pressure to the ideal, in the absence of interionic attraction. This ratio is the osmotic activity coefficient,  $\gamma$ . It may be expressed as

$$\gamma = \frac{\Delta}{(V)\Delta_0}$$

in which  $\Delta$  and  $\Delta_0$  are the freezing point depressions of an electrolyte and of an ideal solute of the same molar concentration, respectively, and  $V$  is the number of ions into which the electrolyte dissociates. The value of the coefficient of an electrolyte, approaching unity at infinite dilution, diminishes with increasing concentration and increasing valence of the ions.

The effective osmotic activity of the urines was always less than their total osmolality. The deviation was slight when mannitol was the predominant solute and small with monovalent electrolytes but assumed considerable magnitude in urines containing high concentrations of multivalent electrolytes.

A plot of the calculated total osmolality of urine versus urine flow is shown in figure 2A. The line shown in the graph describes the regression of plasma osmolality on urine flow during mannitol loading. It was calculated from the values for the total plasma osmolality and urine flow of 28 corresponding periods.



The points for total osmolarity of the urine, obtained after loading with the multivalent electrolytes, sodium sulfate, sodium thiosulfate and sodium ferrocyanide, lie consistently above those for monovalent electrolytes and mannitol. In order to test the possibility that these deviations could be accounted for by differences in osmotic activity, the effective osmotic activity was plotted versus urine flow. It is evident from figure 2B, which presents this plot, that a much closer grouping of the points was obtained. One may conclude, therefore, that the process by which urine is concentrated in the kidney during osmotic diuresis is dependent on the osmotic pressure of the urine rather than on its actual content of solute particles.

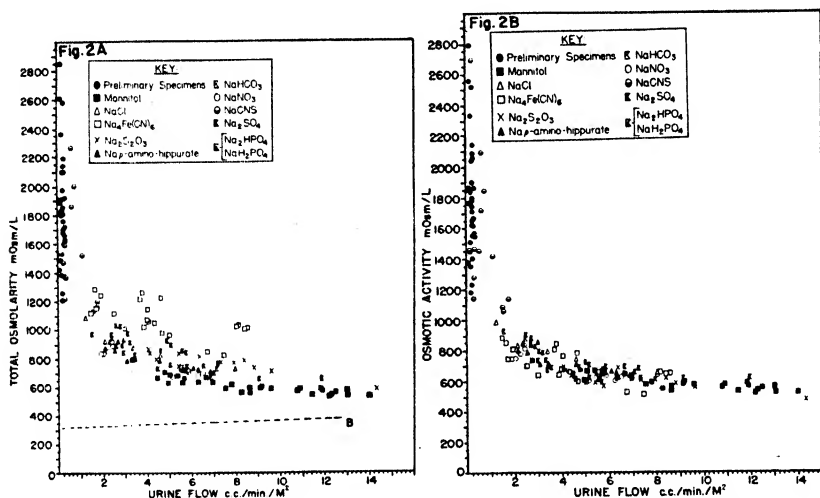


FIG. 2A. TOTAL OSMOLARITY OF URINE VERSUS URINE FLOW. Data for 35 preliminary and 136 post-loading periods. Line B, representing the regression of plasma osmolarity on urine flow for the mannitol experiments, is expressed by the equation  $B = 319.3 + (4.63 \pm 1.26) \text{ urine flow}$ . After loading with solutes more extensively reabsorbed by the tubule, a more rapid increase of plasma osmolarity with increasing flow would be expected.

FIG. 2B. OSMOTIC ACTIVITY OF URINE VERSUS URINE FLOW.

In figure 3A urine flow is plotted against the urinary load (the product of total osmolarity and urine flow). As before, considerable deviation of the points for multivalent electrolytes from the general pattern in the direction of lower flows for a given load is apparent. Again, the discrepancy may be accounted for by the lower osmotic activity of the multivalent electrolytes. The deviation disappears, as shown in figure 3B, when urine flow is plotted versus effective osmotic load (the product of the osmotic activity of the urine and urine flow) which is equivalent to the number of osmotically effective particles excreted per minute. The close grouping of the points indicates that during osmotic diuresis a regular and reproducible relation exists between urine flow and osmotic load, independent of the nature of mode of excretion of the urinary solutes.

The general pattern of decreasing osmolarity with increasing flow (figures 2A

and 2B) is similar to that previously demonstrated for man (2) but close comparison reveals important differences. It will be noted that in the dog, over the whole range of urine flows, urinary concentration is greater than in man. Also, the rate of fall of osmolarity at low flows ( $< 4.0$  cc/min.) is greater and at high flows ( $> 9.0$  cc/min.) less than in man.<sup>2</sup> For man, an equation was derived expressing urine flow in terms of load, based on the assumptions that the rate of change of urine osmolarity with respect to load was proportional to load and that urine osmolarity would asymptotically approach that of plasma. The validity of the hypothesis was tested by the rectilinearity of the plot of the logarithm of the difference between urine and plasma osmolarity versus load. In the dog, attempts to apply such a simple logarithmic expression failed. Different values of plasma osmolarity as well as an expanded formula taking into account the increase of plasma osmolarity with increased

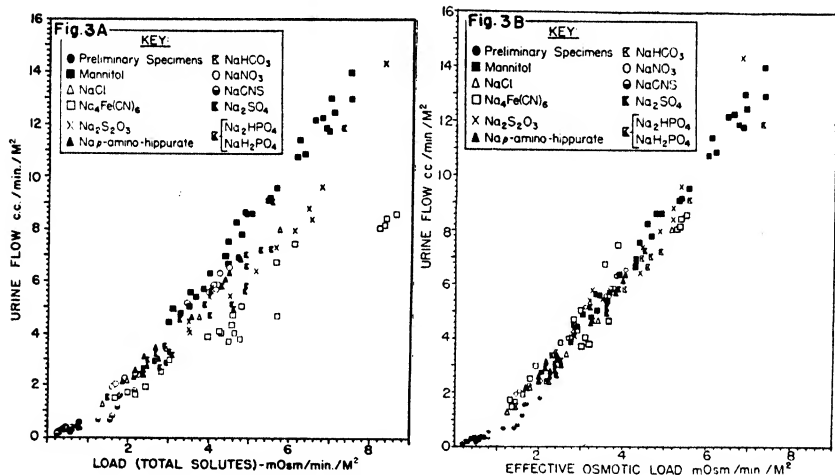


FIG. 3A. URINE FLOW VERSUS LOAD.  
FIG. 3B. URINE FLOW VERSUS EFFECTIVE OSMOTIC LOAD.

loading (figure 2A, line B) were used but yielded curvilinear logarithmic plots. It would appear then, both from the circumstances here described and from the lack of correlation between osmolarity and flow in the range of minimal urine volumes, that the simple hypothesis previously derived will need considerable amplification or revision.

How a hypertonic urine of nearly constant osmolarity is maintained at high rates of flow is a matter of conjecture.<sup>3</sup> If one assumes that water absorption in the

<sup>2</sup> In 2 experiments, not presented here, large loads of mannitol were imposed on hydropenic, anesthetized dogs, by continuous infusion in amounts similar to those used by Wesson and Anslow (15). Urine osmolarity slowly fell as urine flow increased to 30 cc/minute. A regression line calculated for 27 periods of mannitol experiments with flows above 9.0 cc/minute showed that effective osmotic activity diminished by 3.38 milliosmols/l/cc. increase of urine flow.

<sup>3</sup> Wesson and Anslow (15) have presented data on freezing points of one experiment which indicate hypotonicity of urine during mannitol diuresis. In other experiments calculated values for

distal tubule becomes a negligible factor, it would follow that the urine in the proximal tubule is hypertonic to plasma, contrary to generally accepted theories of urine formation. An alternative and perhaps more acceptable explanation may be based on the capacity (ceiling) of the distal tubule either to perform work or to reabsorb water.

*Water Economy.* The excretion of a hypertonic urine during osmotic diuresis allows clearance of the solutes from the body with a saving of water. The water economized is returned to the body and distributed among the body fluids, allowing them to return eventually to their pre-loading tonicity.

The quantity of solutes cleared from the body may be expressed in terms of a solute clearance, calculated in a manner similar to other clearances, as the volume of body fluids which would contain the urinary solutes excreted per minute (load). Mathematically expressed,

$$\text{Solute clearance} = \frac{\text{Load}}{\text{Plasma osmolarity}}$$

Load, the minute excretion of solutes, is the product of osmolarity and urine volume ( $UV$ ), and is equivalent to the  $UV$  of other clearance calculations. The renal water economy is the difference between the solute clearance and the final urine volume:

$$\text{Water economy} = \frac{\text{Load}}{\text{Plasma osmolarity}} - UV$$

Given the assumptions discussed previously (2), one may estimate the proportions of water saved in the proximal and distal tubule. If urine in the proximal tubule and plasma have the same activity, the value of the solute clearance is identical with the volume of isotonic urine leaving the proximal tubule. If, as in the case of urines containing large amounts of multivalent ions, the activity of the urine is lower than that of plasma, an isotonic urine will contain a higher particulate concentration. The volume of urine of a concentration iso-particulate with plasma represents the solute clearance; the differences between this calculated iso-particulate volume and the isotonic volume represents proximal water economy produced by decreased osmotic activity of the tubular fluid. The difference between the total water economized and that economized in the proximal tubule represents distal water reabsorption.

It was considered of interest to relate water economy to urine flow in the present experiments. A graph of this relation is shown in figure 4A, in which total water

the osmolarity of glomerular filtrate and reabsorbate also suggested to them a hypotonic urine. It would appear from the data listed for the control periods that their dogs could not have been in a truly hydropenic condition. The 2 control urine flows listed, 4.6 and 1.1 cc/minute are greatly in excess of truly hydropenic levels and the creatinine  $U/P$  ratios far below the maximum values described (16). One wonders whether the dogs referred to as thirsting for 15 hours had not received water with their exogenous creatinine. Also it is to be noted that the authors did not determine urine urea which would have contributed significantly to the urine osmolarity. It would lead too far to discuss at length to what extent these circumstances affect the conclusions derived from these experiments. At any rate, the pattern of freezing points reported for one experiment (fig. 4). suggest that a water diuresis might have been proceeding concurrently with an osmotic diuresis.

economy is plotted against urine flow. It may be seen that in the case of monovalent electrolytes and mannitol, water economy increased to values of 6.0 to 8.0 cc/minute. The water economy is greater than in man, in keeping with the higher urine osmolarity of the dog. For urines in which multivalent electrolytes predominated, the water economy was greater, approaching values of 14 cc/minute. This deviation is based, as previously pointed out, on the lower osmotic activity of multivalent ions which permits a greater number of particles to be excreted for a given urine flow. Figure 4B presents a graph of the distal water economy based on the considerations discussed in the previous paragraph. It may be seen that a difference between multivalent electrolytes and other solutes no longer is apparent. A value of 6 to 8 cc/minute is reached for all solutes at flows exceeding 7.0 cc/minute with little change at higher flows.

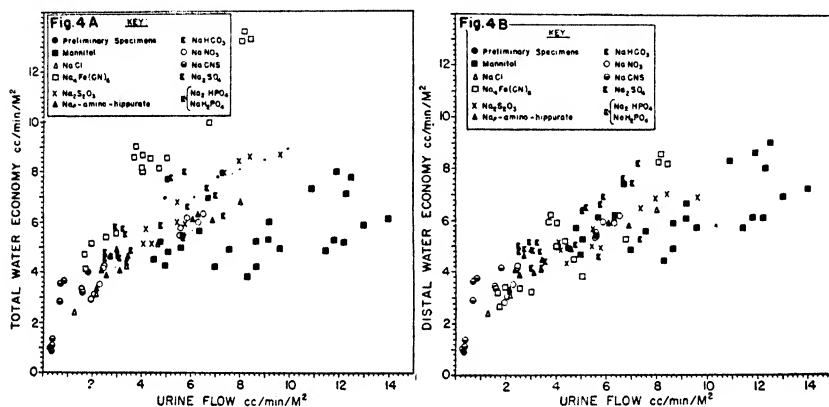


FIG. 4A. TOTAL WATER ECONOMY versus urine flow.

FIG. 4B. DISTAL WATER ECONOMY versus urine flow.

*Solute Composition of Urine and Plasma During Osmotic Diuresis.* The plasma sodium levels increased greatly after loading with multivalent electrolytes reaching 184 to 212 milliosmols/l. Similar increases were noted after sodium chloride and in one sodium bicarbonate experiment. With these 2 exceptions, the monovalent electrolytes produced less marked increases in plasma sodium.

The concentrations of the solute constituents of the plasma, other than those contained in the loading solute, tended to diminish during the course of the experiments. This effect presumably was largely due to shifts of water from the intracellular space. In the case of potassium, however, the fall in plasma level was often greater than could be accounted for by dilution of the extracellular space. Such a fall occurred in all the experiments with bicarbonate, para-aminohippurate, thiosulfate and sulfate and in the first part of the phosphate experiment but was not consistently observed with the other electrolytes. With mannitol the level often increased. In the experiments reported by Flock *et al.* (17) a fall in potassium was also consistent after bicarbonate injection but not after sodium chloride.

The urinary concentrations of the loading solutes varied considerably. The urinary mannitol concentration after loading tended to be higher in the dog than in man, averaging about 350 milliosmols/l. as compared with 330 in man. The anions of the loading solutes were less well concentrated than mannitol. The most striking example was thiocyanate, the urine concentration of which did not rise above 19 milliosmols/l. The average concentrations of the other anions varied from 150 milliosmols/l. for ferro-cyanide to 310 for chloride.

Since a detailed description of the effects of the loading solutes on the patterns of excretion of sodium, potassium and chloride is planned for presentation in separate papers, only a brief description is given here. The urine concentrations of both sodium and chloride during mannitol loading remained relatively constant at a value of about 50 milliosmols/l., independent of the rate of urine flow. Thus the rates of loss of these ions increased with rising urine flow as described previously by others (15, 18). A similar pattern was observed in man (3). Loading with the multi-valent sodium salts produced marked increases in urine sodium concentration, reaching a level of 690 milliosmols/l. in one experiment with ferrocyanide. Impressive effects were also observed on urine chloride concentration after loading with the various electrolytes. Thiocyanate and nitrate increased urine chloride concentration to levels considerably greater than those in the preliminary urines while para-aminohippurate, phosphate, sulfate, thiosulfate and ferrocyanide greatly depressed it. The effect of bicarbonate on urine chloride was similar to that of mannitol.

Urine potassium concentration also remained at a relatively constant level during mannitol loading, the value averaging about 12 milliosmols/l. As has been observed many times previously, the injection of sodium salts increased the rate of potassium excretion.

Urine  $\text{CO}_2$  content, determined in at least one experiment with each loading solute, tended to increase with rising urine flow. The increase was most marked in the ferrocyanide experiment shown in table 2, the value rising from 2 milliosmols/l. in the first post-injection period to 31 in the last period. The main deviation from this pattern was observed with sodium nitrate and with sodium thiocyanate. In these experiments, urine  $\text{CO}_2$  diminished with increasing urine flow, the effect being most marked with nitrate.

In general, the urinary concentrations of sulfate, phosphate and urea diminished with increasing urine flow. Sulfate, present in relatively low concentration in 2 mannitol experiments not presented and in the sodium chloride experiment, was excreted in considerable amount after the injection of thiosulfate. Urinary phosphate concentration, also low after mannitol loading, reached a relatively high value after para-aminohippurate injection, as described previously in man (19).

A comparison of the cation and anion equivalence of the determined urinary electrolytes indicates reasonable agreement in most experiments. With sodium thiocyanate, chloride, sulfate and bicarbonate, however, the cations consistently exceeded the anions. In further studies with thiocyanate, both phosphate and sulfate were found to be excreted in large amounts as shown in the experiment presented in table 2. Phosphate clearance increased 5-fold after the injection of thiocyanate while that of sulfate increased nearly 4-fold. In the case of sodium chloride and

sodium sulfate, the anion not accounted for is most likely phosphate since the other common anions were determined. More detailed studies of these inter-ionic effects during electrolyte loading are in progress.

Glomerular filtration rate and renal plasma flow were measured by creatinine and PAH clearances in 3 mannitol loading experiments. The osmotic diuresis did not affect either function. A more detailed report of the data will be presented in a separate paper.

#### DISCUSSION

The data here presented confirm in a general way the results of previous studies in man (1, 2). Independent of the nature of the solutes and their mode of excretion, with widely varying composition of solute load, an identical relation between urine flow and total solute load obtains. The use of multivalent electrolytes has brought out the fact that it is the osmotic pressure and not the number of particles in the tubular fluid which determines the water reabsorption in the distal tubule during osmotic diuresis. These observations represent important auxiliary evidence that the distal tubule is in fact an osmotic machine not responsive to the chemical but only to the osmotic properties of the tubular fluid.

The simple formula relating flow to load derived for man has been found inapplicable in the dog. It remains for further studies over an even wider range of flows to provide adequate experimental material to test other possible formulations.

The extreme variations in urinary electrolyte concentrations produced, varying from 25 to 690 milliosmols/l. for sodium, from less than 1.0 to 367 milliosmols/l. for chloride, not to mention the variations in the concentration of the loading solutes,—all within the framework of a constant flow-load relation—suggest the proximal tubule as the site of ionic transfer. This exchange must be, to a large extent, independent of the rate of glomerular filtration or urine flow. The patterns and the forces governing electrolyte control in the proximal tubule still remain an unsolved problem. Elsewhere a preliminary analysis of the possible role of the electrical properties of the ions and of the tubular membrane will be presented.

#### SUMMARY

The relation between solute excretion and urine flow of the normal dog kidney was studied under 'resting' hydropenic conditions and during osmotic diuresis. Under resting conditions, urine osmolarity varied from 1200 to 2800 milliosmols/l., independent of the rate of urine flow, while the percentage composition of the urine remained relatively constant. The results suggest the existence of a minimum of urine flow, independent of wide variations in urinary osmolarity. Osmotic diuresis was produced with 10 different loading solutes. They were mannitol, sodium thiocyanate, sodium chloride, sodium nitrate, sodium bicarbonate, sodium para-aminohippurate, sodium sulfate, sodium thiosulfate, sodium phosphate and sodium ferrocyanide. The results confirmed the conclusions previously derived for man, in indicating that urine flow during osmotic diuresis is independent of the chemical nature or mode of excretion of the urinary solutes, and that distal to a spatially distinct point in the renal tubule the percentage composition of the urine remains

unchanged and only water is reabsorbed. It is shown in the present paper that the effective osmotic load (the product of the effective osmotic activity of the urine and urine flow) rather than the actual number of solutes excreted per minute determines the urine flow. The rate of water reabsorption in the distal tubule or the distal water economy increases with increasing urine flow to a value of 6 to 8 cc/minute/ $m^2$  at flows in excess of 7.0 cc/minute/ $m^2$ . The decrease in osmotic activity of urines containing multivalent electrolytes during passage in the proximal tubule results in an additional water economy in this portion of the nephron.

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# EXCRETION OF SODIUM AND POTASSIUM DURING OSMOTIC DIURESIS IN THE HYDROPENIC DOG

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**D**IFFERENT approaches and concepts have been applied to the problem of renal control of electrolyte excretion. Earlier workers were concerned primarily with defining the limiting concentrations of urinary solutes and thought to have fixed certain maximum values for the urinary electrolytes. The question as to whether these maxima were independent of the presence of other solutes in high concentration was resolved by later work which demonstrated that an overall osmotic ceiling limited the concentrations of individual solutes (1, 2).

Another series of studies stems from the concept of a renal threshold (Cushny) existing for certain substances, including all electrolytes. According to this theory, the excretion of a threshold substance is governed by its plasma level. This concept has largely been discarded in consequence of the firm establishment of the glomerular filtration theory, according to which all electrolytes are filtered independent of their plasma level. Modifications of the threshold concept have been proposed (3, 4).

Much work has been based on a consideration of the amounts of electrolytes filtered in the glomerulus and reabsorbed in the tubule. Two main conclusions were drawn by investigators following this line of endeavor: *a*) that the amount of electrolyte reabsorbed is dependent on the filtration rate (5, 6)<sup>2</sup> and *b*) that the reabsorption of sodium and chloride occurs in both proximal and distal tubules in proportions of 85 and 15 per cent, respectively (7).

Recently evidence has been adduced that at least in the case of potassium, tubular secretion may contribute to urinary electrolyte excretion (8, 9), further complicating the picture of renal control of electrolytes.

Studies on osmotic diuresis in hydropenic dogs produced by the sodium salts of a variety of acids have offered the opportunity to study the excretion of electrolytes under standard conditions. Other papers in this series (10, 11) have dealt with the

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<sup>2</sup> The concept proposed by Pitts and his co-workers does not materially differ from the hypothesis of a threshold. A threshold may be defined as a limiting concentration  $C_K$  in either plasma or glomerular filtrate below which the solute is completely reabsorbed. The concept of Pitts, a constant rate of reabsorption per 100 ml. glomerular filtrate, may be expressed  $\frac{C_K \times GFR}{GFR}$ . It is evident that this expression, by cancelling the  $GFR$  term, reduces itself to the expression for a threshold. Whether one assumes complete proportionality of absorption to glomerular filtration or complete independence from it becomes immaterial.



over-all osmotic relations, and the antagonism among the anions. The present report considers the excretion of sodium and potassium under these conditions. Sodium thiocyanate, nitrate, chloride, bicarbonate, *p*-aminohippurate, phosphate, sulfate, thiosulfate and ferrocyanide were employed, as well as mannitol as a reference non-electrolyte.

### EXPERIMENTAL

The experimental conditions, procedures and methods have been described elsewhere (10).

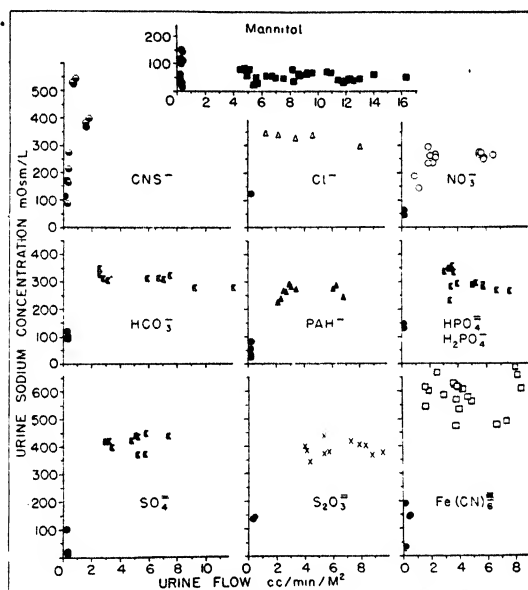


Fig. 1

### RESULTS

Graphs 1 to 4 present the data on the concentration and excretion of sodium and potassium during osmotic diuresis in hydropenic dogs. Considering first the sodium concentration (fig. 1) it is apparent that in the mannitol experiments, a stable urinary level was reached independent of the sodium concentration in the preliminary periods or of the urine flow produced. The plot resembles closely the pattern of chloride excretion during mannitol loading (11). Turning to the electrolyte panels, it is noteworthy that with all univalent ions, with exception of CNS, maximum levels of 200 to 350 mM of sodium per liter of urine were reached, the concentrations tending to decline with increasing flows. In the experiments with the divalent sulfate and thiosulfate, sodium concentrations of 450 and in those with the tetravalent ferrocyanide of 690 mM per liter were obtained. Thiocyanate loading led to variable sodium

concentrations, reaching values of 530 at low flows and of 400 mm per liter at high flows.

Figure 2 presents the data on the amount of sodium excreted per minute. As to be expected from the data of figure 1, which showed little change of concentration with variation in flow, a nearly rectilinear relation between sodium loss and urine flow may be observed.

Turning to the data on potassium, it may be seen from figure 3, which portrays the concentration changes, that with mannitol a low and constant urinary value was obtained. The data on the electrolytes were variable. In some instances, as with thiocyanate and ferrocyanide, an actual increase above preliminary levels was observed. In all cases the concentration decreased at high urine flows, while at inter-

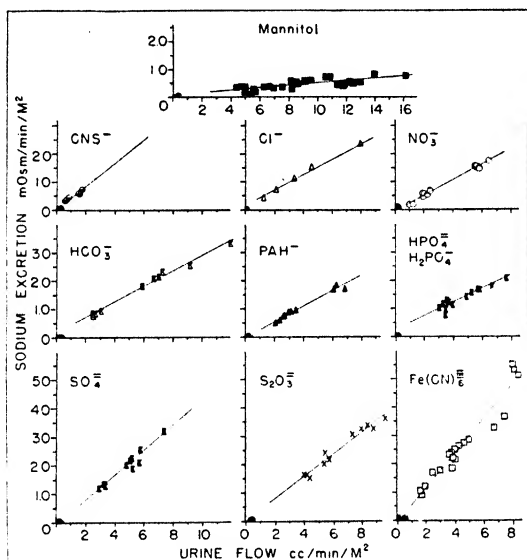


Fig. 2

mediate flows relatively high levels were maintained. In figure 4 is shown the excretion of potassium. It may be seen that even with mannitol, an increased potassium loss occurred at high urine flows. The few data with mannitol at moderate flows of about 4 cc. per minute, show values only little above the preliminary level in agreement with earlier studies in man (12). All the sodium salts showed considerable increases in loss of potassium and great individual variations, with the highest values in the ferrocyanide and phosphate experiments.

Table 1 presents a comparison between the amounts of cations excreted and filtered, observed in the ferrocyanide experiment which showed the greatest excretion of sodium and potassium. The glomerular filtration rate was assessed by the ferrocyanide clearance. It may be seen that the excretion of potassium reached a value of 95 per cent, and that of sodium of 33 per cent, of the amounts filtered. In other ex-

periments the excretion of potassium varied from 40 to 70 per cent, and that of sodium from 10 to 23 per cent of the quantity filtered.

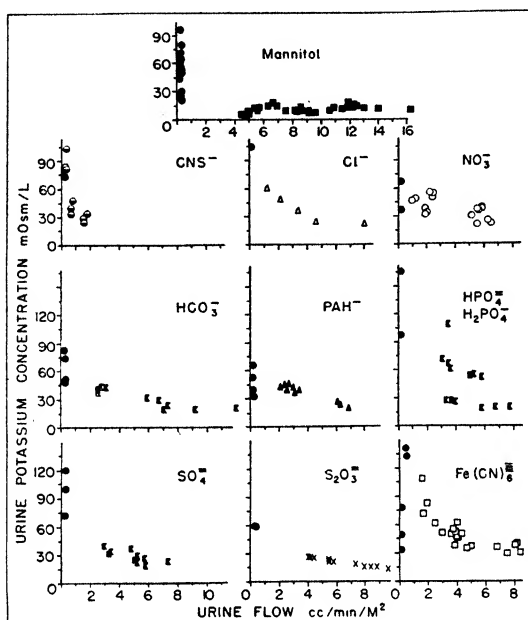


Fig. 3

TABLE I. COMPARISON OF AMOUNTS OF Na AND K EXCRETED AND FILTERED DURING FERROCYANIDE LOADING  
Dog *Pe.*, 16.6 kg., S.A. 0.64 M<sup>2</sup>

PERIOD NO.	CONCURRENT TIME	URINE FLOW	K				Na				CLEAR- ANCE Fe (CN) <sub>6</sub>
			Urine Conc.	Plasma Conc.	Clearance		Urine Conc.	Plasma Conc.	Clearance		
					cc/min/ m. <sup>3</sup>	% GFR			cc/min/ m. <sup>3</sup>	% GFR	
	min.	cc/min/ m. <sup>3</sup>	mm./l.	mm./l.	cc/min/ m. <sup>3</sup>	% GFR	mm./l.	mm./l.	cc/min/ m. <sup>3</sup>	% GFR	cc/min/ m. <sup>3</sup>
P-2	-27 to -3	.50	141.5	4.32	16.4		146	160.0	.5		
	0 to 11	64.4 cc. 18.8% Na <sub>4</sub> Fe(CN) <sub>6</sub> Maintenance 0.65 cc/min.									
1	25 to 35	4.04	61.5	4.17	59.6	84	535	187.5	11.5	16	71.0
2	35 to 45	3.70	50.0	3.96	46.7	67	630	187.5	12.4	18	70.2
3	45 to 55	3.78	55.0	3.99	52.1	69	625	192.0	12.3	16	75.6
	55 to 66	64.4 cc. 18.8% Na <sub>4</sub> Fe(CN) <sub>6</sub> Maintenance 1.30 cc/min.									
4	75 to 85	8.42	30.0	4.14	61.0	77	610	210.0	24.5	31	79.7
5	85 to 95	8.06	39.0	4.17	75.4	95	690	210.0	26.5	33	79.4
6	95 to 105	8.16	40.0	4.26	76.6	94	660	207.0	26.0	32	81.3

## DISCUSSION

The results presented are of interest in relation to several aspects of electrolyte excretion. First, they indicate clearly the lack of an absolute ceiling for the urinary

sodium concentration. Although the levels during loading with univalent ions are comparable to the presumed maxima of the literature, the data indicate that the urinary sodium concentration is also a function of the valence of the anions excreted. Maintenance of electroneutrality appears to be a limiting factor for the urinary concentrations of electrolytes. The somewhat higher sodium levels with CNS, compared with univalent ions, are probably related to the low urinary flows and the outpouring of phosphate and sulfate, so that one may consider the net effect of CNS to be a mixed loading with mono- and divalent ions (10).

A comparison of the excretions of sodium and chloride indicates that although the movements of the two ions parallel each other during non-electrolyte loading, as

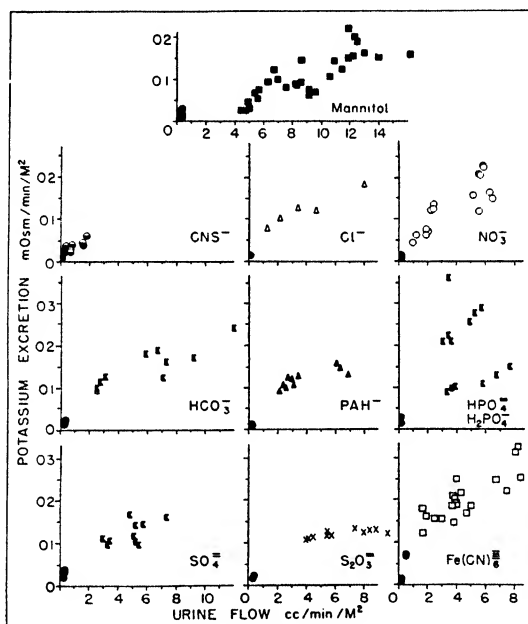


Fig. 4

indicated by the data on mannitol, considerable independence obtains under conditions of electrolyte loading. The difference is most clearly illustrated in the case of ferrocyanide, where urinary concentrations of less than 5 mM per liter and excretions of less than 0.05 mM per minute for chloride are co-existent with concentrations of 690 mM per liter and excretions of 5 mM per minute for sodium, a more than hundred-fold difference.

In regard to the relation between the amounts excreted and filtered of sodium and potassium, it is apparent that in the case of potassium, filtration barely exceeded excretion. The data are in keeping with the evidence presented in favor of potassium secretion (8, 9). For sodium, the high percentage excreted, similar to that found during urea diuresis (13), is added evidence against the assumption of a specific distal mecha-

nism dealing with 15 to 20 per cent of the amount filtered. It should be emphasized that all the variations in the electrolyte concentrations observed take place within the limits of the flow-load relation, and are in agreement with the theory that the proximal tubule is the site of assembly of the urinary load.

#### SUMMARY

The excretion of sodium and potassium of hydropenic dogs was studied during osmotic diuresis produced by sodium thiocyanate, nitrate, chloride, bicarbonate, *p*-aminohippurate, phosphate, sulfate, thiosulfate and ferrocyanide, and mannitol. The urinary concentrations of sodium increased with the valence of the loading anion, and reached a value of 690 mM in the case of the tetravalent ferrocyanide. The sodium clearance rose to as much as 33 per cent of the amount filtered. Potassium excretion increased with rising urine flow in all experiments and accounted for as much as 95 per cent of the amount filtered.

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# OXYGEN TENSION AND pH OF THE RENAL CORTEX IN ACUTE ISCHEMIA AND CHRONIC HYPERTENSION<sup>1</sup>

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ALTHOUGH many theories have been developed as to the mechanism of the hypertension which results from partial renal ischemia, little is known of the actual changes which have occurred in the kidneys. Levy, Light and Blalock have studied the renal arterio-venous oxygen and carbon dioxide differences in hypertensive dogs and have found little change from normal (1, 2); the oxygen uptake of kidney slices were apparently normal (3). Although renal blood flow as measured indirectly by the clearance of para-aminohippurate has been found to be either normal or only moderately reduced after partial renal artery constriction, blood pressure and resistance distal to a Goldblatt clamp have not been extensively investigated. Studies on metabolic alterations in ischemic kidneys are on the whole conflicting (4-6). The present work reports an attempt to measure changes of oxygen tension and pH in the renal cortex of dogs during acute unilateral renal ischemia and in chronic renal hypertension. The same changes observed following acute partial constriction of a renal artery were found in chronic hypertension; they varied in general with the degree of hypertension.

## METHODS

Oxygen tension and pH were measured by electrodes applied to the surface of the renal cortex. Dogs were anaesthetized with intravenous sodium pentobarbital, 30 mg/kg. of body weight. For acute experiments both kidneys were exteriorized and an adjustable clamp placed about the left renal artery, the right being used as a control. This clamp could be adjusted from a distance to avoid handling and possible trauma and approximate degrees of constriction measured on a scale. The amount of constriction was graded 1, 2, 3 and 4 in increasing degrees of severity, 4 being complete occlusion. The kidneys were kept warm and moist by means of saline packs. Acute neurogenic hypertension was produced either by increasing intracranial pressure by saline injection into the cisterna magna, or by bilateral vagotomy and isolation of both carotid sinuses which were then clamped above and below in the usual fashion. Chronic hypertensive dogs were prepared by exteriorizing the left kidney under the skin of the flank and constricting the renal artery with a Goldblatt clamp one month to one year before the experiment. For recording oxygen tension and pH changes, the right kidney was also exteriorized and electrodes were placed upon each kidney, the cortex of the previously prepared left being exposed through a small skin incision.

The oxygen tension electrode first used *in vivo* by Davies and Brink (7) and modified by Davis,

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McCulloch and Roseman (8) was utilized with a slight modification. The electrode itself was connected to a DC amplifier and changes recorded either by a Brush inkwriter or an electroencephalographic amplifier with a Grass inkwriter; in some experiments values were obtained by direct reading of a coupled microvoltmeter. An electrode modified after that developed by Nims (9) was employed to measure pH; it consisted of a half cell with a thin glass membrane surrounded by a silver-silver chloride half cell in saline agar. Measurements were made with the same apparatus used for oxygen tension. Both electrodes were approximately 8 mm. in diameter (fig. 1). The pH electrode rested upon the surface of the renal cortex beneath the capsule, measuring in actual units the fluid immediately under the cell. In using the oxygen tension electrode it was necessary to puncture the cortex by the platinum wire (no. 28) to a distance of approximately 2 mm. The electrodes were maintained in position by means of small plastic holders loosely tied to the kidney under its capsule by figure-of-eight ligatures

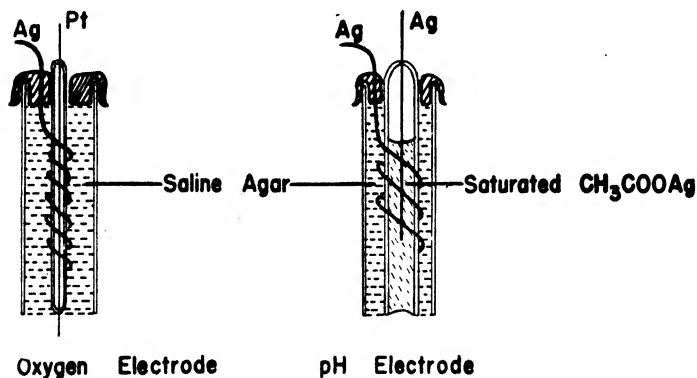


FIG. 1

without compression or trauma to the kidney. Constriction of the renal vein was avoided and care taken to prevent trauma to the kidney during the entire procedure. In a few instances the left kidney was exteriorized to a location beneath the skin one to several weeks before the experiment. In every case the 'normal' kidney was compared to the 'ischemic' kidney, electrodes being interchanged to negate differences in individual electrodes. While the values obtained for pH were in absolute units, those for oxygen tension were only relative.<sup>3</sup>

## RESULTS

*Effect of Acute Constriction of a Renal Artery Upon Oxygen Tension and pH.* Eight normal dogs were used; in some cases the kidneys had been exteriorized previously and in others they had not. In no case were there significant differences either in oxygen tension or pH between the 2 kidneys before partial arterial constriction. When the clamp was tightened to degree 1 or 2, an abrupt fall in oxygen tension occurred which was followed after a few minutes by a gradual rise toward the control value. Further degrees of clamping produced sustained lowering of the

<sup>3</sup> Salomon (10) plotted the current flowing through an unknown between a negative platinum point and a large non-polarizable electrode. When the current is plotted as a function of the voltage there appears a series of plateaus, the lowest of which is determined by the reaction:  $2\text{H}^+ + \text{O}_2 + 2 \text{ electrons} \rightarrow \text{H}_2\text{O}_2$ . As long as the voltage applied is that corresponding to the plateau, the current is a measure of the oxygen tension. In these experiments 0.9 of a volt was applied from a potentiometer through a series resistor of one megohm.

oxygen tension in the cortex. Upon release of the clamp the oxygen tension returned to normal values. During severe degrees of constriction, a slight increase was noted in the opposite unaffected kidney which slowly returned to normal after release of the arterial clamp (fig. 2, table 1).

About one or 2 minutes after partial arterial constriction, the pH of the cortex altered consistently in the acid direction. These changes were of considerable magnitude, amounting in some cases to 0.3 or 0.4 of a pH unit, and lasting as long as the clamp was applied without further change. After release of the clamp the pH slowly returned to control values. Complete occlusion caused further change in the same direction. No variation occurred in the opposite kidney (table 1).

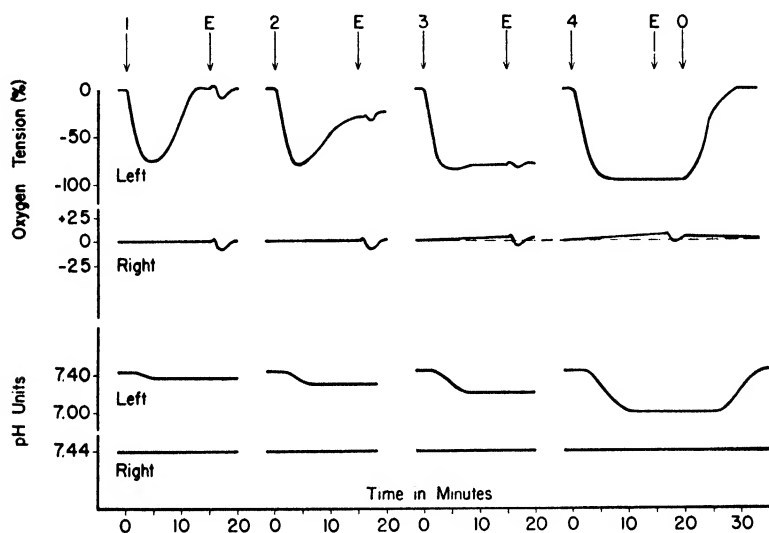


FIG. 2. CHANGES IN OXYGEN TENSION AND pH OCCURRING IN RENAL CORTX WITH VARYING DEGREES OF CLAMPING AND RESPONSE TO EPINEPHRINE. 1, 2, 3 and 4: Increasing degrees of constriction of left renal artery, 4 being complete occlusion. O: Left renal artery completely unclamped. E: Intravenous injection of 10  $\mu$ g. epinephrine.

The intravenous injection of 10  $\mu$ g. of epinephrine before renal constriction produced a consistent fall of approximately 9 per cent in the oxygen tension which rapidly returned to normal; the effect was always over in about 5 minutes. An attempt was made to determine whether the ischemic kidney was more sensitive to the action of epinephrine than normally as far as oxygen tension was concerned, as has been reported for renal blood flow (11). This was not the case, the ischemic kidney reacting less than the contralateral one. The greater the degree of clamping the less was epinephrine effective in lowering the oxygen tension in the clamped kidney. Its action was completely abolished by complete occlusion of the renal artery. No changes in pH were observed.

*Changes in Oxygen Tension and pH of the Renal Cortex in Chronic Hypertensive*



*Dogs.* Five dogs were used. Although one of them (0-51) had been prepared some 3 months previously no significant elevation of blood pressure was observed at

TABLE 1. CHANGES IN OXYGEN TENSION AND pH OF RENAL CORTX WITH VARYING DEGREES OF CLAMPING

DEGREE OF CLAMPING	O <sub>2</sub> TENSION CHANGE FROM BASE		pH		MAXIMUM FALL AFTER 10 $\mu$ g. EPINEPHRINE <sup>1</sup>
	5 min.	15 min.	5 min.	15 min.	
	$\frac{v_o}{\%}$	$\frac{v_o}{\%}$			$\%$
<i>Left Kidney</i>					
0	0 (+3.1-4.0)	0 (+3.0-4.0)	7.44 (7.42-7.45)	7.44 (7.42-7.45)	9.4 (8.9-10.2)
1	-80.0 (76.1-83.0)	0.0 (+2.1-3.3)	7.40 (7.39-7.45)	7.40 (7.38-7.44)	9.0 (8.6-9.8)
2	-78.0 (76.0-86.0)	-27.1 (25.1-29.2)	7.31 (7.29-7.37)	7.28 (7.25-7.31)	7.6 (7.1-8.2)
3	-76.1 (75.6-88.2)	-74.8 (70.8-76.2)	7.13 (7.02-7.22)	7.12 (7.02-7.22)	2.9 (2.1-4.3)
4	-93.1 (82.7-97.6)	-93.8 (83.1-97.6)	7.01 (6.92-7.20)	7.01 (6.92-7.20)	0.0 (0.0-0.2)
<i>Right Kidney</i>					
0	0 (+3.1-4.0)	0 (+3.0-4.0)	7.44 (7.42-7.45)	7.44 (7.42-7.45)	9.4 (8.9-10.2)
1	0.0 (0.0-0.0)	0.0 (0.0-0.0)	7.44 (7.43-7.45)	7.44 (7.43-7.45)	9.4 (8.9-10.0)
2	0.0 (0.0-0.3)	+1.1 (0.0-2.0)	7.45 (7.42-7.46)	7.45 (7.42-7.46)	9.5 (9.0-10.4)
3	+0.8 (0.0-1.1)	+1.6 (0.0-2.6)	7.45 (7.42-7.46)	7.45 (7.42-7.46)	9.4 (8.8-11.1)
4	+3.6 (0.8-5.1)	+3.8 (1.1-5.9)	7.44 (7.42-7.46)	7.44 (7.42-7.46)	9.5 (9.1-12.2)

Results shown are averages of 8 normal dogs. Figures in parentheses are the maximum and minimum values obtained. Both kidneys were exteriorized and an adjustable clamp placed around the left renal artery. Oxygen tension and/or pH electrodes were placed on both kidneys. Oxygen tension and pH were measured on both kidneys following varying degrees of constriction (4 being complete occlusion and 0 being no constriction). The response to 10 $\mu$ g. of epinephrine was measured 15 minutes following clamping.

<sup>1</sup> Fall in oxygen tension.

any time after unilateral renal arterial constriction. One other (0-42) developed a slight elevation of blood pressure. In the others a consistent rise in the mean arterial pressure following operation was obtained. It was necessary to select those which attained persistent hypertension; the usual event after unilateral arterial

constriction was a transient hypertension lasting a few weeks or months. Therefore the dog which had little or no elevation of blood pressure was used as a control. Table 2 shows the changes which were observed in both kidneys. There was an alteration of the pH in the acid direction of approximately 0.15 to 0.24 of a unit in the 3 hypertensive dogs, a slight change in the dog with slight hypertension and none in the dog which had no hypertension. The oxygen tension was decreased in the constricted kidney roughly in proportion to the degree of hypertension, being 21 to 45 per cent lower in the 3 hypertensive dogs with little change in the 2 where hypertension had not been established. The pH of the opposite cortex was of the same value as that found in normal dogs.

There was a definite decrease in the response of the ischemic kidney to intravenous epinephrine as compared with the contralateral one. This change was roughly in proportion to the hypertension.

TABLE 2. CHANGES IN OXYGEN TENSION, pH, AND RESPONSE TO EPINEPHRINE IN DOGS WITH EXPERIMENTAL RENAL HYPERTENSION

DOG	AVERAGE CONTROL B.P. (MEAN)	POST- OPERATIVE AVERAGE B.P. (MEAN)	DURATION OF HYPERTENSION	pH		O <sub>2</sub> TENSION DECREASE IN LEFT	MAXIMUM FALL OF O <sub>2</sub> TENSION AFTER 10 µg. EPINEPHRINE	
				Left	Right		Left	Right
	mm. Hg	mm. Hg				%	%	%
0-5	144	172	60 days	7.27	7.46	45	2.1	12.1
0-12		164	1 yr.	7.20	7.44	33	2.0	9.8
0-38	136	152	30 days	7.30	7.45	21	3.2	9.5
0-42	148	157 <sup>2</sup>	38 days	7.39	7.46	5	8.9	10.1
0-51 <sup>1</sup>	142	146	3 mo.	7.45	7.46	2	9.4	9.4

The dogs were operated on by constricting the left renal artery with a Goldblatt clamp. pH is given in absolute units and oxygen tension as per cent decrease in left kidney from the 'control' right kidney.

<sup>1</sup> No significant elevation of blood pressure at any time. At autopsy right kidney weighed 56.1 gm. and left kidney 37.9 gm.

<sup>2</sup> Dog recovering from hypertension. Final blood pressure recorded was 152 mm. Hg.

*Changes in Oxygen Tension and pH of the Renal Cortex in Acute Neurogenic Hypertension.* Two experiments were successful. The blood pressure rose in one case from 106 to 160 mm. Hg and the other from 90 to 180 mm. Hg, returning to approximately control values after the pressure in the cisterna magna was released. In both cases the oxygen tension became lower by about 10 to 13 per cent and returned to normal levels as the blood pressure fell. This experiment was duplicated in one dog. During cerebral ischemia no renal changes in pH occurred. In 2 dogs bilateral vagotomy and isolation and clamping of both carotid sinuses caused no significant reproducible changes in pH or oxygen tension. Variations in oxygen tension which occurred appeared to be more or less haphazard, possibly affected by alterations in respiratory rate and volume rather than to the elevation of blood pressure.

*Effects of Miscellaneous Drugs.* Four drugs were chosen in order to determine whether pH or oxygen tension of the ischemic kidney could be effected, although

no planned series of experiments was made. In normal animals adenosine triphosphate, 8 mg. intravenously, produced an initial rise and then a fall below control values of about 15 per cent. In the chronic dog which did not develop hypertension the injection of adenosine triphosphate increased the oxygen tension in both kidneys about 35 per cent; in the other animal with slight hypertension about 15 per cent elevation occurred. The increase lasted 5 to 10 minutes. In a hypertensive dog a decrease in oxygen tension of 9 per cent was observed in the unaffected kidney lasting about 5 minutes, with no effect upon the ischemic kidney. Tetraethylammonium chloride (40 mg.) and tyramine hydrochloride (one mg.) had no effect on oxygen tension or pH in either the constricted or the opposite kidney in acute experiments. Isoamylamine (100 mg.) transiently increased the oxygen tension of normal kidneys about 25 per cent, followed by a decrease to below normal and a return to the base line in about 15 minutes. In hypertensive dogs similar but smaller changes were observed in the constricted kidney with a fall of about 20 per cent in the unconstricted one. This experiment was repeated 3 times with consistent results. Ciba 5968 (hydrazino-phthalazine hydrochloride, 20 mg.) caused a decrease in the normal kidney followed by an increase with return to control values. After moderate renal arterial constriction no effect was observed on the clamped kidney but the response was exaggerated in the contralateral one.

#### DISCUSSION

The results of these experiments indicate that there is a consistent alteration of pH of the cortex of the kidney in the acid direction during acute renal ischemia and in chronic renal arterial constriction accompanied by elevation of the blood pressure. The data also show that acute renal ischemia, as might be expected, lowers the oxygen tension of the renal cortex. Chronic renal hypertension is also accompanied by an alteration in oxygen tension as compared with the opposite kidney. As measurements of oxygen tension were in relative values, one kidney could only be compared with the other; therefore it was not known whether or not alterations occurred in the opposite unaffected kidney. The pH electrode, however, gave absolute values and changes of great magnitude were consistently observed. It is difficult to explain this finding. If changes occurred in the whole kidney there should be changes in the pH of the urine which would be followed rapidly by alterations in the acid-base balance of the body serious enough to cause death of the animal. Some restoration of pH must have occurred at an area other than the surface of the kidney. These electrodes, being about 8 mm. in diameter, probably gave a representative sampling of that portion of the renal cortex directly under them. It is difficult to believe that these alterations occurred in extra-renal fluid not related to metabolic functions of the renal cortex itself. Measurements of pH were consistent and constant in normal kidneys and were consistently more acidic in those with renal ischemia. It would be important to follow changes in more discrete areas of the kidney with micro-electrodes inserted into the cortex at some distance.

The changes in pH suggest that in areas adjacent to the cortex marked alterations in acid-base balance accompany ischemia, in a direction found in anoxia, which possibly involve cellular functions. The fact that the pH of the urine is es-

entially normal under these conditions suggests that some change occurred in the nephron with more acid produced at a cortical level and possibly a readjustment at a subcortical level. It is also conceivable that local cortical vasoconstriction occurred. Acidification of the cortex, however, was not wholly dependent upon hypoxia. With only mild degrees of clamping the readjustment of oxygen tension to control values was still accompanied by a decided change of pH toward the acid side, giving rise to a situation of slight renal ischemia with normal oxygen tension and an acid pH.

The reaction of the blood flow to partial constriction of the renal artery has been described previously (11). In short, vasodilatation occurs apparently with a restoration of total blood flow to normal levels. Subsequent degrees of clamping of the renal artery eventually produces a state in which renal blood flow is consistently lowered. Similar changes were seen in oxygen tension. For example a slight degree of clamping caused an 80 per cent fall in oxygen tension which was restored in 15 minutes to normal values. These changes occurred slowly. Further constriction produced a similar fall in oxygen tension with a rise to only 27 per cent below control values. Changes in oxygen tension observed can probably be explained by changes in renal blood flow not measured in these experiments, although other factors may be important. The close correlation between pH and oxygen tension suggests that one affects the other. It would be more likely for local hypoxia to affect pH than vice versa. During ischemia, changes in pH were not measurable for one to 2 minutes after the clamp was tightened and the oxygen tension had become reduced. The lag in the pH and the degree of its alteration suggest a profound local renal metabolic disturbance. Measurement of pH and oxygen tension on the cerebral cortex has also demonstrated parallelism in response to drug-induced convulsions and histotoxic anoxia (12). It appears probable, however, that the renal effects are concerned in some way with the development of experimental hypertension in animals.

It is well known that epinephrine causes a transient decrease in renal blood flow mainly by efferent arteriolar constriction. When small doses are employed, the effect is usually over in a few minutes. The fact that epinephrine altered oxygen tension probably indicates a change of blood flow. The fact that epinephrine did not alter pH would suggest that there is no common denominator between pH and oxygen tension or the effects were too transient to alter the pH. The increased sensitivity of the ischemic kidney to the vasoconstrictor action of epinephrine, previously described for blood flow (11) was not observed for oxygen tension. In no case did the sensitivity of the renal vascular bed to epinephrine (as measured by oxygen tension) increase following partial constriction of the renal artery.

In the dogs with acute neurogenic hypertension the effects were slight or absent. It is possible that the fall in oxygen tension observed during acute cerebral ischemia was secondary to alterations in the respiratory rate. In one experiment respirations temporarily ceased for a short interval. The fact that acute hypertension produced by carotid sinus clamping was not accompanied by consistent changes in the oxygen tension suggests that renal vasoconstriction in proportion to the vasoconstriction elsewhere occurred. None of the drugs used appeared to increase the

oxygen tension or pH in the ischemic kidney by an action which could be considered intrinsic to the mechanism involved in ischemia. Although Ciba 5968 has been said to increase renal blood flow in normal animals and in normal and hypertensive human beings (13), it did not affect oxygen tension in acute experiments under the conditions obtained. The only increases noted were after adenosine triphosphate in the non hypertensive Goldblatt dogs. However, the daily injection of adenosine triphosphate has been found to exert a chronic hypotensive effect in renal hypertensive dogs (14).

The validity and reliability of the methods should be considered. The measurements of pH appeared to be consistent and varied little from dog to dog, changing in the same direction in every experiment involving both acute and chronic renal ischemia. The electrodes were checked constantly *in vitro* and gave absolute values accurate when compared with a standard glass electrode. The pH of the un-operated kidney in chronic experiments varied only 0.02 of a unit from one dog to another and was remarkably constant in normal animals. These observations suggest that the changes observed with ischemia, while marked and difficult to explain, are real.

Measurements of oxygen tension were relative, merely comparing the output of emf from the electrode in one kidney with the same electrode in the other kidney. The sensitivity was such that respiratory variations of approximately 2 per cent were discernible; when respiratory rate was slow a definite lag occurred with a rise a few seconds after inspiration and expiration followed by a slow fall. Although to our knowledge this is the first time this electrode has been used in the cortex of the kidney, it has been used previously, both in its macro and micro form, in the cortex of the cerebrum and cerebellum with reproducible results. Complete renal arterial obstruction by the clamp caused a decrease of about 94 per cent in the oxygen tension of the ischemic kidney as compared to the opposite one, suggesting that the changes observed were real. Kidneys were constantly observed to determine whether or not renal venous constriction and congestion were present; in no case were such conditions observed. The kidneys remained normal in color and consistency when exteriorized and wrapped in warm saline packs. Chronic exteriorization of a kidney did not change its oxygen tension or pH when no Goldblatt clamp had been applied.

It is unlikely that diminished pulsations of the kidney itself resulting from the application of a clamp affected the measurements; under no conditions was the pulse detected by the electrodes.

The ischemic kidney was compared with the contralateral one in the chronic experiments. A possible but not probable explanation for the changes observed is that the unaffected kidney had increased its oxygen tension, therefore showing a relative decrease in the 'ischemic' kidney. The degree of clamping necessary to produce chronic hypertension was estimated according to this scale at between 2 and 3. The relative decrease in oxygen tension appeared to be somewhat less in the chronic hypertensive dogs than would be obtained in an acute experiment with a similar degree of clamping. This was also true for pH. It is possible that the increase in blood pressure following unilateral renal arterial constriction compensated in part for the reduction in blood flow and therefore minimized the changes.

## SUMMARY

Experiments have been performed to determine the pH and relative oxygen tension of the renal cortex in acute renal ischemia and in chronic renal hypertension in dogs anaesthetized with Nembutal. In normal dogs progressive degrees of unilateral renal arterial constriction caused decreases in oxygen tension and marked acidity in the ischemic kidney. In chronic renal hypertensive dogs there was a change of pH in the acid direction and a decrease in oxygen tension of the renal cortex of the ischemic kidney as compared with the opposite one. Small doses of epinephrine appeared to cause less decrease in the oxygen tension of the renal cortex in both the chronic and acute ischemic kidney than in the contralateral kidney. In acute experiments oxygen tension first fell and then rose to normal levels with slight constriction of the renal artery. Under these conditions pH remained acidic.

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# EFFECT OF DIETARY RESTRICTION OF SALT AND PROTEIN ON BLOOD PRESSURE OF HYPERTENSIVE RATS<sup>1</sup>

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THE effect of a restriction of sodium chloride in the diet upon the blood pressure in experimental hypertension has been a subject of contradictory reports. Grollman and Harrison (1) reported that various diets which contained very little sodium chloride brought about a decrease in the average systolic pressure of rats from 157 mm. Hg to 128 mm. within 4 days after the diet was started. In rats fed diets composed exclusively of potato or peanut or soy bean, the pressure which ranged between 165 and 180 mm. within 6 days fell to 120 to 130 mm. When the various diets contained 0.5 per cent NaCl, the fall in pressure was partially prevented, and with 2 per cent NaCl no fall in pressure occurred. This hypertension had been produced by the application of Grollman's figure-of-eight ligature to the kidney, combined with unilateral nephrectomy in some cases. Dick and Schwartz (2) fed dogs, which had been made hypertensive by the injection of streptococci, the Kempner rice diet and reported a striking reduction of pressure in 10 of 11 dogs within 2 months. Page and Lewis (3) have reviewed the literature concerning the effect of diet upon experimental hypertension and reported that in their experiments with 2 dogs which were made hypertensive by wrapping the kidneys in silk, feeding a diet furnishing 25 mg. of Na per day for 8 to 9 months, caused no significant change in arterial pressure even though a marked loss in weight occurred. Handler and Bernheim (4) have reported higher blood pressures for hypertensive rats eating diets rich in casein. Restriction of calories to the level of maintenance "resulted in a fall to virtually normal pressures on all diets but that containing an unusually excessive amount of salt." Removal of salt from diets containing 8 per cent casein, "lowered systolic pressure to actually subnormal values." Salt removal from a high protein diet only slightly reduced blood pressure. "Addition of excess salt to a low protein ration produced an impressive hypertension." However, their values for systolic blood pressure in the rat are very low in comparison to the findings of other workers (5).

## METHODS

Hypertension was induced in Sprague-Dawley rats, weighing 70 to 130 gm. by wrapping the kidneys with silk (6). If the silk was applied with the proper tension,

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the rat gradually developed hypertension over a period of 2 to 4 weeks and remained in apparent good health for a number of months. When the silk was too tight, hypertension of 200 mm. Hg or more developed within a few days and the rat lived only 3 to 4 weeks. If it was too loose, no hypertension developed.

The systolic pressure of the rat was determined with the apparatus and technique devised by Byrom and Wilson (7) with the incorporation of some of the modifications of Williams *et al.* (8). The tail was sealed in the plethysmograph with a mixture of vaseline, beeswax and paraffin. Arising from the plethysmograph which was filled with water, was a capillary tube of the bore suggested by Shuler *et al.* (9). The narrow pressure cuff, recommended by Shuler *et al.* was connected to a source of compressed air through an adjustable reducing valve. Enclosing the plethysmograph was a cylinder through which water at 43°C. was circulated, as recommended by Sobin (5). Only the tail of the rat was warmed for at least 20 minutes before readings of pressure were attempted. Six consecutive pressure readings with a maximum variation of 10 mm. Hg were taken as the systolic pressure for any particular determination.

The systolic blood pressure of normal rats in our studies ranged between 145 and 165 mm. Hg and repeated determinations on the same rat on successive days gave values with the maximum variation rarely exceeding 15 mm. Hg. This range of systolic pressure in normal rats confirms the range of 154 to 157 mm. Hg which has been reported by Sobin (5). Systolic pressures of 180 mm. Hg or more were considered hypertensive. In hypertensive rats, wide fluctuations in pressure occurred, commonly as much as 60 mm. in one week. This variation made repeated pressure determinations over a considerable period of time imperative, in order to properly evaluate the level of pressure. Although no attempt was made to space regularly the pressure determinations in an individual rat over the experimental course, at least one determination was made in each 10-day period. The pressures determined in each 10-day period were averaged. These values for each period were in turn averaged to give the values designated as the average systolic pressure of tables 1 and 2. In this way the time element could be introduced into the data, providing a more reliable index to actual pressure trends and ascribing proportionally less value to multiple pressure readings, taken within a 10-day period, that might be recorded inadvertently at either extreme of the wide variation of pressure observed in hypertensive rats.

*Diets.* The rats were fed a commercial ration<sup>4</sup> designated *diet 4*, during the period of development of hypertension. The diets consisted of the following: *Diet 1*: polished rice 56, sucrose 44, vitamins A, D, thiamine, riboflavin, calcium pantothenate and nicotinic acid. *Diet 2*: polished rice 56, sucrose 44, vitamins given in *diet 1*, plus pyridoxine; *diet 3*: sucrose 95.2, casein<sup>5</sup> 4.3, lard 0.2 and salt mixture<sup>6</sup> 0.3, vitamins. This diet approximated the carbohydrate, fat, protein and inorganic contents of *diets 1* and *2*. *Diet 5*: sucrose 95, casein 4, lard 1, thiamine, riboflavin Ca pantothenate, nicotinic acid. *Diet 6*: sucrose 77, casein 18, lard 5, salt mixture 0.3, vitamins. *Diet 7*: sucrose 75, casein 18, lard 5, salt mixture 2, vitamins. *Diet 8*: boiled

<sup>4</sup> Dickinson dog chow.

<sup>5</sup> 'Vitamin-free' casein from Nutritional Biochemicals.

<sup>6</sup> Phillips and Hart salt mixture. *J. Biol. Chem.* 109: 657, 1935.



potato. *Diet 9*: sucrose 75, casein 18, lard 5, salt mixture with NaCl omitted 1.5, vitamins. Vitamins were given as 2 drops of halibut-liver oil per week per animal and thiamine 3 mg., riboflavin 3 mg., pyridoxine 3 mg., Ca pantothenate 20 mg.,

TABLE 1. EFFECT OF RICE DIETS ON EXPERIMENTAL HYPERTENSION

RAT	OBSERVATION PERIOD					EXPERIMENTAL PERIOD I (40 DAYS)					EXPERIMENTAL PERIOD II (40 DAYS)				
	Diet	Average Systolic Blood Pressure	Pressure Range	No. of Determinations	Length of Period	Diet	Average Systolic Blood Pressure	Change in Average Pressure	Pressure Range	No. of Determinations	Average Systolic Blood Pressure	Change in Average Pressure	Pressure Range	No. of Determinations	
		mm. Hg	mm. Hg		days		mm. Hg	mm. Hg	mm. Hg		mm. Hg	mm. Hg	mm. Hg		
1	4, Control	194	182-208	4	24	1, Rice	190	-4	157-220	6	176	-18	171-179	3	
2		196	187-205	2	14		177	-19	151-193	6	181	-15	154-200	3	
3		185	154-211	7	34		187	2	172-205	6	213	28	163-238	3	
4		202	168-235	6	20		189	-13	160-214	5	178	-24	155-197	6	
5		196	186-204	4	20		171	-25	148-184	5	166	-30	145-190	6	
6		244	236-253	5	30		206	-38	189-231	4	214	-30	180-267	6	
7		209	200-220	4	12	2, Rice	194	-15	159-204	4	208	-1	193-253	9	
8		182	172-197	4	14		198	16	178-222	4	216	34	175-246	8	
9		193	173-212	6	39	3, Purified	194	1	166-208	5	180	-13	166-207	9	
10		206	187-217	8	42		205	-1	192-215	5	201	-5	187-214	9	
11		182	170-203	6	15		169	-13	151-184	5	170	-12	161-180	9	
12	4, Control	217	196-240	5	20		Died on 33d day								
13		206	194-215	3	20		207	1	207-207	2	Died on 11th day				
14		204	162-281	4	20		189	-15	160-210	7	206	2	206	1	
15		197	192-201	2	20		176	-21	155-203	3	186	-11	170-194	5	
16		218	200-224	3	20		223	5	203-258	5	238	20	214-275	4	
17		200	200	1	20		213	13	173-268	4	210	10	192-233	5	
18		196	183-204	3	20		191	-5	180-215	6	197	1	183-211	4	
19		188	185-192	2	20		180	-8	143-204	5	190	2	177-219	3	
20		227	216-238	2	20		244	17	221-269	3	Died on 24th day				
21		221	219-222	2	20		243	22	225-255	3	Died on 30th day				

nicotinic acid 25 mg., *p*-amino benzoic acid 300 mg. and choline 1000 mg. per kg. of ration. The rats were kept in cages with screen bottoms.

## RESULTS

The effect of the various diets on the systolic pressure is seen in tables 1 and 2. Four of 6 rats eating the rice diet (*diet 1*) showed a significant drop in pressure which became manifest in the first 40-day period and persisted through the succeeding period. Of the 2 rats, (1 and 3) showing no significant change in pressure during the first 40-day period, one showed a drop in pressure and one a very marked increase

in pressure, in the second 40-day period. Of the 2 rats eating *diet 2* which differed from *diet 1* solely in its content of pyridoxine, only *rat 7* showed a drop in average pressure and this occurred only in the first experimental period. However, the maintenance of a hypertensive level in these 2 rats might be for the same unknown

TABLE 2. EFFECT OF SALT RESTRICTION ON EXPERIMENTAL HYPERTENSION

RAT	OBSERVATION PERIOD				EXPERIMENTAL PERIOD I (40 DAYS)				EXPERIMENTAL PERIOD II (16 to 40 DAYS)						
	Diet	Av. Systolic Blood Pressure	Pressure Range	No. of Determinations	Length of Period	Diet	Av. Systolic Blood Pressure	Change in Av. Pressure	Pressure Range	No. of Determinations	Av. Systolic Blood Pressure	Change in Av. Pressure	Pressure Range	No. of Determinations	Length of Period
		mm. Hg	mm. Hg				days	mm. Hg	mm. Hg		mm. Hg	mm. Hg	mm. Hg		
22	4, Control	198	169-258	10	49	5, 95%	204	6	171-236	5	165	-33	152-207	4	40
23		235	216-257	4	20	sugar,	218	-17	199-242	5	193	-42	167-205	3	24 <sup>1</sup>
24		184	155-203	10	99	no salt	191	7	161-219	5	183	-1	171-191	4	40
25		203	181-236	9	76	6, .3%	181	-22	177-186	3	186	-17	174-194	5	40
26	7, Control	203	182-237	9	96	salt, 18	192	-11	186-202	3	188	-15	164-212	5	40
27		197	186-208	5	37	% protein	177	-20	164-184	3	172	-25	150-193	6	40
28		228	196-268	8	60		251	23	235-263	4	248	20	229-261	5	40
29		207	191-222	6	52		180	-27	170-192	4	200	-7	174-205	4	40
30	8, Potato	205	182-225	10	95		207	2	190-230	4	233	28	204-253	4	40
31		246	218-260	5	44		Died on 7th day <sup>1</sup>								
32		210	192-230	5	43		195	-15	180-221	4	213	3	183-250	4	40
33		187	169-212	5	34		178	-9	169-184	4	195	8	171-242	4	40
34	9, No NaCl	198	184-206	6	42		191	-7	177-208	4	252	54	200-289	4	40
35		221	184-266	4	21		204	-17	170-248	9	195	-26	182-208	2	16
36		189	180-195	4	20		217	28	179-245	9	186	-3	179-192	2	16
37		208	160-218	4	20		181	-27	156-219	9	190	-18	184-195	2	16
38		204	186-231	4	21		179	-25	150-224	9	153	-51	150-155	2	16
39		211	188-245	3	19		177	-34	156-220	9	219	8	219	1	16 <sup>1</sup>
40		196	191-200	2	9		188	-8	164-220	9	167	-29	162-172	2	16
41		198	192-213	4	35		166	-32	154-176	3	190	-9	163-218	5	42
42		199	180-232	4	35		208	9	177-240	3	207	8	207	1	18 <sup>2</sup>
43		212	181-256	4	35		194	-18	164-212	3	171	-41	145-200	3	42

<sup>1</sup> Died during course of experiment.

<sup>2</sup> Was accidentally killed during course of experiment.

reason as in the case of *rat 3* on *diet 1*. With *diet 3* an attempt was made to approximate the carbohydrate, protein, fat and ash content of the rice diet with sucrose, casein, lard and an inorganic salt mixture. The rats eating this diet, showed some drop in pressure but not as marked as obtained with *diet 1*, the rice diet. It might be pointed out that in the rats eating *diets 1, 2* and *3*, the pressure did not drop to

a normal level for more than one reading. The lowest average obtained in the case of one rat was 166 mm. Hg. On the other hand, the above results in spite of their inconsistency seem more valuable when compared with those of the control rats. During the first 40 days of the experimental period, only 2 of the 9 control rats showed a significant drop in average pressure and this persisted in one rat for the succeeding 40-day period. With the other controls, in contrast to the results obtained with *diets 1* and *3*, the pressure either continued at the same level of hypertension or was aggravated. Control rats *16*, *18* and *19* were studied concurrently with the rats on *diets 1* and *3*.

The experiments, whose data are listed in table 2, were expected to show the effect of 3 factors in the diet, namely salt mixture, sodium chloride and protein. These 3 factors are at minimal values in *diet 5*. With this diet a significant decrease in pressure occurred in one rat (no. 23) during the first 40-day period and in 2 (nos. 22 and 23) of the 3 rats in the second period. This diet differs from *diet 3* in the absence of salt mixture and appeared to have a greater effect in lowering the pressure but its results approximated those of *diet 1* (rice) which differed in its source of protein and inorganic matter. The effect of elevating the protein from 4 per cent to 18 per cent, plus the salt mixture supplement at 0.3 per cent, is seen in the results with *diet 6*. A comparison of these with *diets 1* and *3* (table 1) indicate that the protein content was of little or no significance, although the actual lowering of pressure was slightly greater with the 4 per cent protein diet. The effect of elevating the salt mixture to 2 per cent with 18 per cent protein is seen in the results with *diet 7*. In the second 40-day period the average pressure not only failed to decrease but 2 of the rats (nos. 30 and 34) had an increase to the maximal range which we have observed. The effect of a dietary elimination of NaCl with the protein estimated at 2 per cent, and with protein at 18 per cent casein is seen in *diets 8* and *9*, respectively. The removal of sodium chloride appeared to result in the greatest decrease in pressure during the first 40 days of any of the diets used. This confirms the report (1) that NaCl must be almost absent from the diet to permit the hypotensive effect of such diets. However the time required for such effect was much longer than previously reported. In the succeeding period the hypotensive effect remained for 5 of the 9 rats. The diets with NaCl omitted had about the same effect in lowering pressure as the rice, *diet 1*.

The decrease in systolic pressure which occurred in rats fed diets restricted in NaCl was not associated with a significant loss of weight. Of the 28 rats fed such diets only 7 showed a loss of weight and in 6 of these the loss was less than 10 per cent. All of the other rats including the controls showed a gain in weight with the exception of 2 animals.

As shown in table 3, surgical removal of the silk capsule from the kidneys of hypertensive rats markedly lowered the pressure in 3 of 4 animals. This confirms a report of Page (6) for dogs. In these 3 rats, the hypertension was associated with the presence of the silk capsule and was not likely due to any increased activity of the sympathetic nerves. Etamon chloride in doses of 5 mg. and 25 mg. per kg. of body weight in one hypertensive rat had no effect on its blood pressure within 10 minutes

of intra-peritoneal injection. Pentobarbital sodium, to a degree of surgical anesthesia did not reduce the blood pressure in another hypertensive rat.

All diets which contained no more than 0.05 per cent NaCl or no NaCl at all enabled the rats to live significantly longer than the control diets 4 and 7, which contained the usual dietary amount of sodium chloride. Only 40 per cent of the 15 rats eating control diets lived 220 days post-operatively whereas 83 per cent of 24 rats eating diets restricted in NaCl lived this span. This observation is a confirmation of Grollman and Harrison although our rats eating either the control or restricted diets seemed to live longer than those of the other workers.

TABLE 3. EFFECT ON BLOOD PRESSURE OF REMOVAL OF SILK FROM KIDNEYS

RAT		PRIOR TO REMOVAL	FOLLOWING REMOVAL											
41	Systolic Pressure	206 219 211	169 208 205 217 176 170 196 151 173 169											
	Days	16 9 3	5 11 19 32 41 50 63 77 89 112											
44	Systolic Pressure	171 204 209	158 149 149 158 143 184 151 160											
	Days	16 9 3	5 11 19 32 41 50 58 74											
43	Systolic Pressure	195 198 214	205 207 215 176 196 226 160 184 168 246 167											
	Days	23 9 3	5 11 19 32 41 50 63 77 89 96 110											
24	Systolic Pressure	216 231 194	200 181 212 212 204 191 160 192											
	Days	29 21 4	3 16 27 35 44 53 105 114											

Systolic pressure expressed in mm. Hg.

#### DISCUSSION

Our study indicates that casein in amounts up to 18 per cent of the diet had no demonstrable effect upon hypertension, which finding is in agreement with some previous reports. It disagrees with the report of Handler and Bernheim that systolic pressure was higher with 19 per cent casein in the diet than with 8 per cent. One variable which might explain the contradictory results obtained by various workers of the effect of dietary protein upon experimental hypertension is the degree of renal excretory insufficiency. In animals with inadequate renal excretion, the ingestion of greater amounts of protein might have a hypertensive effect which would be absent in cases of adequate excretion. The decline in blood pressure of our rats fed diets restricted in NaCl was decidedly smaller and required a longer time than that reported by Grollman and Harrison. However, this result contrasts with the failure of Page and Lewis to obtain a lowering of pressure in dogs made hypertensive by the same method as was used with the rats. In considering the role of sodium in the maintenance of renal hypertension it is pertinent to point out that Abrams, De Friez, Fasteson and Landis (10) found that hypertensive rats in their choice of solutions for drinking elected to take one-third to one-half as much NaCl and NaHCO as the normal rats. The dietary restriction of sodium might lower hypertensive

blood pressure indirectly by an effect upon the adrenal cortex, since Dean, Shaw and Greep (11) have reported that such diets induced in the adrenals microscopic changes which they interpreted as indicating increased secretory activity of the cortex.

#### SUMMARY

Hypertension was produced in rats by wrapping silk around both kidneys. Fifteen of 28 hypertensive rats when fed various rations containing between 0.0 to 0.05 per cent sodium chloride showed following a 40-day adjustment period declines in average systolic pressure of 15 to 51 mm. Hg. The pressure never dropped to a normal level for two consecutive readings. Diets with the lowest sodium chloride content had the greater hypotensive effect. This was true irrespective of natural sources of foods such as rice and potato or purified sucrose and casein. With a few exceptions the time required for a significant lowering of systolic pressure was 15 to 30 days which is much longer than previously reported (1). Such diets lowered systolic pressure without any significant loss of weight.

Dietary casein in amounts of 4 and 18 per cent appeared to exert little or no effect on the hypotensive response of such rats to sodium chloride restriction.

Of the 10 hypertensive rats eating the control diets and completing the experiment, none showed a decline of 15 mm. Hg in average pressure and in 4 cases the pressure increased. Only 40 per cent of the control rats lived 220 days post-operatively, whereas 83 per cent of the rats eating diets low in NaCl lived this period. This confirms the work of Grollman and Harrison.

The results obtained upon removal of the encapsulating silk and the injections of Etamon chloride or pentobarbital sodium indicated that the hypertension in these animals depended upon renal rather than nervous factors.

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# EFFECTS OF OXINE, CARBOSTYRIL AND QUINOLINE ON FROG NERVE

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CYANIDE is capable of inhibiting both nerve respiration and conduction (1). Carbon monoxide also produces these effects, and light is able to reverse the CO inhibition (2). These results suggest the possibility that a metal system is involved in nerve activity. It should be possible to put this matter to a direct test through the use of compounds which chelate with metals under physiological conditions. One such compound which is able to combine with a number of metals is 8-hydroxyquinoline or oxine, widely employed in analytical chemistry (3). This substance has been shown (4) to combine with a variety of metals at physiological temperatures and in the physiological range of pH. The metal complexes thus formed are insoluble in aqueous solutions but soluble in organic fluids, especially chloroform. Neither quinoline, nor any of the isomerides of oxine appear to combine with metals under physiological conditions (4).

Oxine thus offers possibilities for use in investigations where the role of metals in physiological processes is under question. This compound is known to possess antibacterial and fungistatic activity and both these actions may be related to the ability of oxine to chelate with metals (5, 6). Oxine has also been reported to inhibit photosynthesis in *Chlorella pyrenoidosa* (7), an effect which is probably associated with the metal-binding action of this compound. The unusual avidity of 8-hydroxyquinoline for iron has been applied by Waring and Werkman (8) to prepare an iron-deficient medium in which, after supplementation, the growth requirements of certain bacteria for iron could be examined.

These considerations led to a decision to study the effects of oxine on frog nerve fibers. By means of techniques which have been described (9, 10) the actions of oxine in conduction block and on the nerve resting potential were examined. The results obtained with oxine have been compared with the actions of 2-hydroxyquinoline (carbostyryl) and of quinoline. The latter two compounds possess no metal-chelating ability under the conditions of these experiments (4).

## RESULTS

*Action of 8-Hydroxyquinoline.* Oxine is only slightly soluble in physiological solutions but enough of it can be dissolved in Ringer's solution to demonstrate a

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definite action on frog nerve. When a 10 to 15 mm.-segment of bullfrog sciatic nerve (with sheath intact) is exposed to a 0.003-molar solution (pH 7.2), cessation of conduction in the *A* group of fibers in this segment takes place in about 30 minutes. This conduction block is readily reversed if the oxine is removed shortly after the compound *A* spike has disappeared, but recovery is incomplete or absent if exposure to the oxine is continued for 15 or more minutes following complete block in all *A* fibers. Reversible block by 8-hydroxyquinoline is illustrated in figure 1 *B* (*open circles*).

In addition to its ability to produce block of conduction, oxine is able to effect a slight shift in the positive direction of the nerve resting potential. This hyperpolarization (*curve 1*, figure 1 *A*) consists of a rapid increase in potential to a level which is then maintained relatively constant until the oxine is washed away, after which the positivity slowly declines. This action of oxine is similar to the hyper-

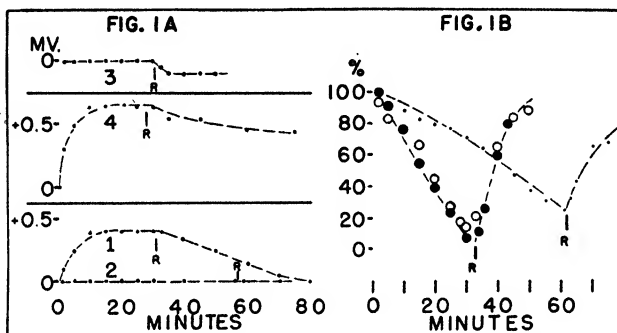


Fig. 1. A. RESTING POTENTIALS. Action of .003 M oxine on living nerve (1) and on dead nerve (2). Action of .007 M carbostyryl (3) and of .007 M quinoline (4). Washing out of nerve segment with Ringer's solution occurred in each case at point indicated with an *R*. Millivolts plotted against minutes. B. Conduction block of *A* fibers. Oxine (.003 M) shown as *open circles*, quinoline (.007 M) as *large points* and carbostyryl as *small points*. Washing again at *R*. The *A* spike height (in % of original level) plotted against minutes.

polarization produced in frog nerve by dilute carbamate solutions (10). The hyperpolarization is an action by the living nerve fibers. Neither a dead nerve (*curve 2*, figure 1 *A*) nor the connective tissue nerve sheath respond in this manner.

*Action of Quinoline.* Quinoline, though not a chelating agent, is nevertheless capable of producing both conduction block and hyperpolarization in frog nerves. The blocking action of a 0.007-molar solution of quinoline (pH 7.2) is shown in figure 1 *B* (*heavy solid points*) while the effect on the resting potential is presented in *curve 4*, figure 1 *A*. The presence of an —OH group in the ring system is therefore unnecessary either for the production of block or for hyperpolarization.

*Action of 2-Hydroxyquinoline.* Carbostyryl fails to chelate with metals, presumably because of its inability to form a 5- or 6-membered ring, co-planar with the other rings. Yet this ineffectiveness of carbostyryl to complex with metals does not prevent it from acting as a nerve blocking agent (figure 1 *B*, *light points*). This similarity in action of oxine and carbostyryl in producing conduction block may be

contrasted with the difference in effectiveness of these two compounds in biological reactions involving the process of metal chelation. Thus, to cite one such case (11) the growth of the fungus, *Phymatotrichum omnivorum* was inhibited by a solution containing oxine at a concentration of 0.5 p.p.m. whereas carbostyryl when present at a concentration of 300 p.p.m. permitted growth.

Of considerable interest, however, is the fact that 2-hydroxyquinoline, unlike both quinoline and oxine, is unable to induce in living nerve an elevation of the resting potential (curve 3, figure 1 A). This is true even when saturated solutions of carbostyryl (pH 7.2) are employed. This lack of effect of 2-hydroxyquinoline appears not to be due to a failure of this compound to reach the nerve fibers because 1) completely desheathed nerves also fail to respond to carbostyryl and 2) nerve block is readily produced by this compound.

#### DISCUSSION

The chief result of this investigation is to show that both the blocking action and the hyperpolarization appear to be unrelated to the process of metal chelation, if such occurs. It is true that oxine is slightly more effective, in that more dilute solutions (about 1/2) of this compound are required than in the case of either quinoline or of carbostyryl. The order of magnitude of the difference, however, is far from what is expected on the basis of the property of metal chelation. This is not to deny that oxine may have effects related to its ability to complex with metals, but this characteristic does not appear to be of primary importance in causing the results herein reported. No dependent relation exists in these three compounds between the ability to block and the hyperpolarizing action. Oxine and quinoline produce both effects, whereas carbostyryl blocks but causes no apparent change in the resting potential.

The results suggest that the nitrogen is of primary significance in producing the positivity. An —OH group is not required. The presence of such a group in the 8-position does not appear to hinder the action of the N atom, but its location in the 2-position does affect the activity of the N. In other words, an —OH group in the quinoline nucleus is unnecessary either for blocking action or for elevation of the resting potential, but an —OH group in the 2-position (and possibly others) will prevent the hyperpolarizing action while leaving unaffected the capacity of the molecule to block conduction.

#### SUMMARY

A comparison of the actions on frog nerves of oxine, a powerful metal-chelating compound, with carbostyryl and quinoline, both non-chelators, has revealed no evidence that either conduction block or hyperpolarization are processes exclusively associated with the property of metal chelation.

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# EFFECT OF LYMPHOID NECROSIS DUE TO NITROGEN MUSTARD AND ROENTGEN IRRADIATION ON NEUROMUSCULAR FUNCTION OF HYPOPHYSECTOMIZED ANIMALS<sup>1</sup>

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AFTER removal of the pituitary gland neuromuscular function is impaired, due mainly to a dysfunction of the nerve element (1). Two measurable components of the dysfunction of neuromuscular system are a decline of action potential during repetitive stimulation (1) of the nerve, a sign of easy fatigability, and a decrease of acetylcholine synthesis by nerve tissue (2). Both the action potential and the acetylcholine synthesis return to normal during administration of adrenocorticotrophic hormone (ACTH) to hypophysectomized animals (2). Hence, it is inferred that the fatigability of nerve is regulated *in vivo* through the ACTH output of the pituitary gland. The mechanism through which ACTH regulates the activity of nerve is not yet identified. It is known, however, that the mass of lymphoid tissue and thymus is reduced during ACTH administration (3, 4). Since there is a demonstrable relationship between the mass of thymus and the activity of nerve (5-10), an attempt was made in the following to reduce the mass of lymphoid tissue and thymus *in vivo* by means other than ACTH and to ascertain whether or not the effect of ACTH in restoring the neuromuscular function in hypophysectomized rats can be duplicated by these other measures.

## METHOD

In the absence of measures with exclusive effect on the mass of lymphoid tissue and thymus the effect of ACTH on the mass of these tissues was approximated or exaggerated by using 2 agents instead of one, namely roentgen irradiation and nitrogen mustard. These 2 measures differ in their side effects from each other and from ACTH. Furthermore, nitrogen mustard and roentgen irradiation were used in low concentrations, and 48 hours elapsed between the last treatment and the experiments to allow the animals time to recover from the reversible transitory effects.

**Roentgen Irradiation.** The animals received 150 r in air by use of 140 kv. at a 25-cm. distance and employing a 3-mm. aluminum shield for 3 minutes twice in a 72-hour interval. The animals were killed 2 days after the second treatment. Low

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concentrations were used in order not to jeopardize the health of the hypophysectomized rats.

*Nitrogen Mustard.* The preparation of Lederle Laboratories was injected endoperitoneally once in concentrations from 0.4 to 2 mg/kg. of body weight. The animals were killed 2 days later.

*Animals.* Approximately 6-week old hypophysectomized rats were used about 2 weeks postoperatively. The completeness of the removal of the pituitary gland was established at autopsy. Unoperated rats served as controls.

*Action Potential Measurements.* Electromyograms were taken during repetitive stimulation of the sciatic nerve by the following method (1, 2). The spinal cord of the rats was destroyed up to the midthoracic region. The 2 legs were used as separate preparations. One leg was firmly fixed at the knee and ankle joint to an animal board. Bakelite-insulated silver electrodes were used. One recording silver wire electrode was inserted into the upper half of the gastrocnemius muscle; the other into the tendon of the gastrocnemius muscle. Stimulating silver wire electrodes, 4 mm. apart, were placed either near or directly on the sciatic nerve. The nerve was stimulated in 10-minute intervals for one minute with 30 and 50 pulses/second, each of 100 microseconds duration and of 'supramaximal' intensity. The sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and muscle action potentials were superimposed on the screen of the cathode ray tube. The action potential was recorded photographically.<sup>2</sup>

The maximum amplitude of the action potential was measured and the values obtained at the beginning of a series of stimuli served as 100 per cent. The amplitude of the action potential taken at 30-second intervals was expressed as percentage of the amplitude of the first action potential occurring at the beginning of stimulation.

*Blood Circulation.* In a few representative experiments upon conclusion of the experiment, Berlin Blue was injected into the abdominal aorta of the rats to ascertain whether or not the blood supply of the gastrocnemius muscle remained adequate during the experiments. Each leg was uniformly discolored within a few seconds indicating that the blood supply was adequate.

*Acetylcholine Synthesis.* The rats were killed by separating the head from the body. The brain was excised, weighed and after finely mincing, the brain samples were incubated for assay of the synthesis of acetylcholine. Because of the lability of the enzyme, not more than 2 minutes were allowed to elapse between the killing of the rats and the beginning of shaking and incubation. The synthesis of acetylcholine was ascertained according to a slightly modified method of Quastel, Tennenbaum and Wheatley (11, 12). Two hundred mg. of minced brain, 3 mg. of physostigmine salicylate, and 2 ml. of a modified mammalian Ringer's solution at pH 7.4 were shaken and were incubated aerobically for 4 hours at 37°C. After incubation the amount of acetylcholine synthesized was assayed biologically on the sensitized *Rectus abdominis* muscle of the frog.

The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of incubated mixtures the acetylcholine content of identical non-incubated mixtures.

<sup>2</sup> The electrical equipment was constructed by the Grass Instrument Co.

*Leucocyte Counts.* Blood samples were drawn avoiding, as much as possible, stress due to handling. The rats were wrapped loosely in a towel, the tail was rubbed with xylol and then heated with a 100-watt light-bulb for about 5 minutes. The tip of the tail was cut off with a sharp razor blade. The first drops of blood were discarded and the following drops were collected with standard white cell blood pipettes. Total white cell counts were made by standard methods, eosinophil cell counts were made following the method described by Speirs and collaborators (13), and differential leucocyte counts were made on blood smears stained with a Hastings solution.

## RESULTS

*Acetylcholine Synthesis.* The amount of acetylcholine synthesized by brains of non-operated control rats averaged  $14.70 \mu\text{g}/100 \text{ mg.}$  brain tissue. Brains ob-

TABLE 1. EFFECT OF NITROGEN MUSTARD AND ROENTGEN IRRADIATION ON ACETYLCHOLINE SYNTHESIS OF BRAIN OF HYPOPHYSECTOMIZED AND NON-OPERATED RATS

TREATMENT	NO. OF EXPER.	AMOUNT OF ACETYLCHOLINE SYNTHESIZED, 100 MG. BRAIN (WET WEIGHT) IN 4 HOURS (FOLLOWED BY THE STANDARD ERROR OF MEAN)		
		$\mu\text{g}$	own control	non-operated control
<i>Non operated Rats</i>				
Control . . . . .	30	$14.70 \pm 0.3$	100	100
Nitrogen mustard inj. (2 mg/kg.)	20	$10.10 \pm 0.6$	130	130
Roentgen irradiated . . . . .	14	$18.64 \pm 0.3$	127	127
<i>Hypophysectomized rats</i>				
Control . . . . .	17	$8.21 \pm 0.6$	100	50
Nitrogen mustard inj. (2 mg/kg.)	6	$7.66 \pm 1.0$	93	53
(0.4 mg/kg.) . . . . .	14	$10.66 \pm 0.7$	130	72
Roentgen irradiated . . . . .	14	$10.51 \pm 0.6$	128	72
ACTH injected (2) . . . . .	16	$13.40 \pm 0.5$	163	91

tained from rats injected with nitrogen mustard or irradiated with roentgen rays synthesized on the average 29 per cent more acetylcholine than the brains obtained from non-operated control rats (table 1). Decreasing the concentration of nitrogen mustard did not significantly increase the ability of brains to synthesize acetylcholine. It is interesting to note that the activity of choline acetylase decreases if directly exposed to the effect of nitrogen mustard (14, 15).

The amount of acetylcholine synthesized by brains of hypophysectomized rats averaged  $8.21 \mu\text{g}/100 \text{ gm.}$  of brain tissue (2). Therefore, the acetylcholine synthesis is on the average 45 per cent less than in non-operated control animals. Roentgen irradiation and administration of nitrogen mustard in low concentrations (0.4 mg/kg.) induced some increase in the ability of brain to synthesize acetylcholine. This increase averaged 29 per cent (table 1). Administration of nitrogen mustard in larger concentrations (2 mg/kg.) was poorly tolerated in hypophysectomized rats and severe symptoms of intoxication occurred. The ability of brain of hypophysectomized ani-

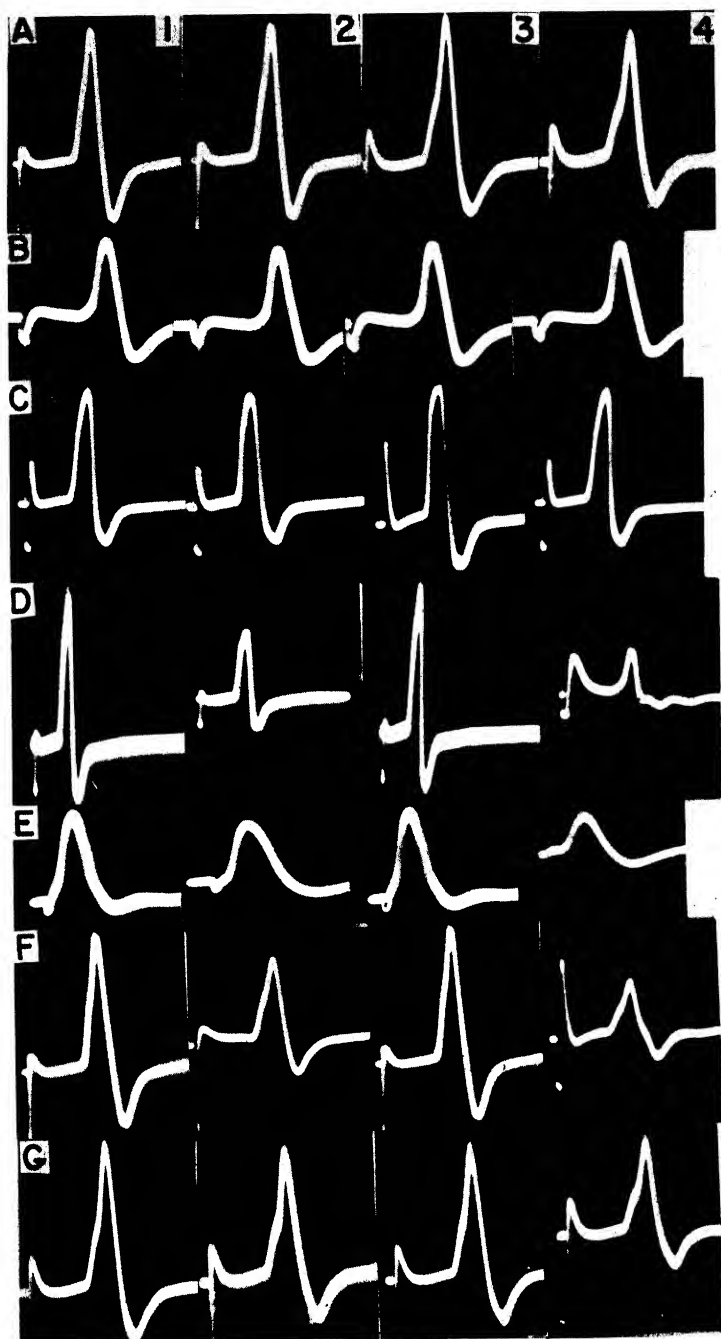


Fig. 1. EFFECT OF NITROGEN MUSTARD AND ROENTGEN IRRADIATION on muscle action potential during repetitive indirect stimulation. *Row A*: non-operated control rat; *Row B*: non-operated nitro-

mals injected with nitrogen mustard in concentrations of 2 mg/kg. did not increase (table 1). Administration of ACTH to hypophysectomized rats increased the ability to synthesize acetylcholine to 100 per cent (2).

**Action Potential Measurements.** In non-operated control rats the amplitude of the muscle action potential during stimulation of the sciatic nerve with 30 pulses/second appeared to be maintained unaltered during a 30-second stimulation period with the apparatus used. The amplitude of action potential decreased about 15 per cent during stimulation with 50 pulses/second for 30 seconds (fig. 1, table 2). In the hypophysectomized control rats the amplitude of the action potential decreased by

TABLE 2. EFFECT OF NITROGEN MUSTARD AND ROENTGEN IRRADIATION ON ACTION POTENTIAL OF HYPOPHYSECTOMIZED AND NON-OPERATED RATS

TREATMENT	NO. OF EXPTS.	AMPLITUDE OF ACTION POTENTIAL TAKEN AT THE END OF A 30 SECOND STIMULATION PERIOD IN PER CENT OF THE AMPLITUDE OF THE FIRST ACTION POTENTIAL (FOLLOWED BY THE STANDARD ERROR OF MEAN)					
		Frequency of stimulus/sec.:					
		30			50		
		first act. pot.	own control	non-operated control	first act. pot.	own control	non-operated control
		%	%	%	%	%	%
<i>Non-operated Rats</i>							
Control.....	30	99 ± 0.5	100	100	85 ± 0.8	100	100
Nitrogen mustard inj. (2 mg./kg.).....	20	102 ± 0.8	103		89 ± 0.6	105	
Roentgen irradiated.....	14	97 ± 0.7	98		87 ± 0.7	102	
<i>Hypophysectomized Rats</i>							
Control.....	17	42 ± 1.9	100	43	28 ± 0.7	100	33
Nitrogen mustard inj. (2 mg./kg.).....	9	44 ± 1.0	107	44	28 ± 0.5	100	33
(0.4 mg./kg.).....	14	56 ± 0.9	133	57	37 ± 0.6	133	43
Roentgen irradiated.....	14	51 ± 1.1	121	52	36 ± 0.3	130	42
<i>Hypophysectomized ACTH-injected Rats (2).....</i>							
	15	84 ± 1.7	200	85	69 ± 1.5	246	81

58 per cent at the end of a 30-second stimulation period with 30 pulses/second and 72 per cent at the end of a 30-second stimulation period with 50 pulses/second (1) (table 2, fig. 1). Administration of nitrogen mustard to hypophysectomized rats in

gen mustard (2 mg/kg.)-injected rat; Row C: non-operated roentgen-irradiated rat; Row D: hypophysectomized control rat; Row E: hypophysectomized nitrogen mustard (0.4 mg/kg.)-injected rat; Row F: hypophysectomized roentgen-irradiated rat; Row G: hypophysectomized ACTH-injected rat (2). Column 1: Action potential records taken at the beginning of a 30-second stimulation period with 30 pulses/second. Column 2: Action potential records taken at the end of a 30-second stimulation period. Column 3: Action potential records taken at the beginning of a 30-second stimulation period with 50 pulses/second. Column 4: Action potential records taken at the end of a 50-second stimulation period with 50 pulses/second.

concentrations of 2 mg/kg. did not improve the ability to maintain the action potential unaltered. Hypophysectomized rats, however, are hypersensitive to nitrogen mustard and the relatively low concentrations of 2 mg/kg. of nitrogen mustard induced already severe symptoms of poisoning. Therefore, the effect on the maintenance of action potential of nitrogen mustard in concentrations lower than 2 mg/kg. was also ascertained. The ability of hypophysectomized rats improved about 30 per cent after administration of nitrogen mustard in concentrations of 0.4 mg/kg. A similar 30 per cent increase was found in hypophysectomized rats after roentgen irradiation (fig. 1, table 2), whereas, the ability to maintain the amplitude of action potential returned to normal in the hypophysectomized rats after administration of ACTH (2).

TABLE 3. EFFECT OF NITROGEN MUSTARD ADMINISTRATION AND ROENTGEN IRRADIATION ON LEUCOCYTE COUNT OF HYPOPHYSECTOMIZED AND NON-OPERATED RATS

TREATMENT	NO. OF EXPER.	TOTAL LEUCOCYTE COUNT <sup>1</sup>		EOSINOPHIL CELL COUNT		LYMPHOCYTE COUNT		NEUTROPHIL CELL COUNT	
		No.	own control	No.	own control	No.	own control	No.	own control
			%		%		%		%
<i>Non-operated Rats</i>									
Control.....	18	11,900	100	205	100	8,490	100	3,210	100
Nitrogen mustard inj. (2 mg./kg.).....	24	8,400	71	84	41	5,880	69	2,436	76
Roentgen irradiated.....	22	2,370	20	28	14	1,170	14	1,170	36
<i>Hypophysectomized Rats</i>									
Control.....	17	23,195	100	146	100	19,810	100	3,240	100
Nitrogen mustard inj. (0.4 mg./kg.).....	10	13,125	57	32	22	10,810	54	2,285	70
Roentgen irradiated.....	17	3,700	16	30	21	2,280	11	1,390	43

<sup>1</sup> The numbers given are average values.

*Leucocyte Counts.* In hypophysectomized rats the leucocyte count was higher than in non-operated control rats due mainly to an increase of the lymphocytes in the peripheral blood. Roentgen irradiation and nitrogen mustard administration decreased the total leucocyte count, the eosinophil cell count, the lymphocyte count, and the neutrophil cell count. A similar percentage decrease was found in both non-operated and hypophysectomized rats due to roentgen irradiation and nitrogen mustard administration. Hypophysectomy increased, however, the sensitivity of the rats to nitrogen mustard since similar percentage decrease in the leucocyte counts was obtained in non-operated rats by administration of concentrations of 2 mg/kg. of body weight of nitrogen mustard and in hypophysectomized rats by administration of 0.4 mg/kg. The absolute number of cells of each group of control, roentgen irradiated, and nitrogen mustard-injected rats within the non-operated and hypophysectomized groups are comparable since counts were made on the same rat before and after roentgen irradiation and nitrogen mustard administration.

## DISCUSSION

In these experiments an attempt was made to decrease the mass of lymphoid tissue and thymus to a degree comparable to or more complete than the decrease induced by administration of ACTH. Since measures decreasing the mass of lymphoid tissue without having side effects are not known, measures were chosen having different side effects. Since the effects of roentgen irradiation and nitrogen mustard administration on the neuromuscular system were about the same but did not duplicate the effects of ACTH in restoring neuromuscular function in hypophysectomized rats, it may be inferred that changes in the mass of lymphoid tissue and thymus induced by the 3 measures are about the same and that the difference in the action of ACTH was due to actions not evoked by either nitrogen mustard or roentgen irradiation.

*Some Known Effects of the Three Measures Used.* Roentgen irradiation, nitrogen mustard, and ACTH affect a variety of tissues and processes that may be relevant to neuromuscular function. Thus all 3 measures reduce the size and induce cytological changes in the thymus and lymphoid tissue (16-23). The lymphoid tissue is affected by roentgen irradiation and ACTH through accelerating the normal rate of physiological aging and death rate of cells, probably through a change in the metabolism of thymonucleoprotein (23). Nitrogen mustard in addition induces an inhibition of the mitotic activity of cells (17, 22).

ACTH in relatively low concentrations cannot directly substitute for acetylcholine, does not inhibit cholinesterase, and increases acetylcholine synthesis mainly by changing the relative concentrations of inhibitor and potentiator agents (24, 25). Nitrogen mustard can substitute for acetylcholine because of its physicochemical properties (22), it inhibits cholinesterase (15, 26) and choline acetylase (14, 15), but in the concentrations used this inhibition is reversible. Furthermore, nitrogen mustard is rapidly decomposed or eliminated and could not have direct effects on either processes forty-eight hours after administration. Roentgen irradiation in the concentrations and manner used has no known effects on acetylcholine metabolism.

*Thymus and Lymphoid Tissue.* Since extracts of thymus, pancreas and liver (8) *in vitro* inhibit the activity of choline acetylase, but similar extracts of lymphoid tissue do not, it appears likely that nitrogen mustard, ACTH and roentgen irradiation induced their effects of acetylcholine synthesis mainly through reduction of the mass of the thymus rather than because of reduction of the mass of lymphoid tissue. Liver and pancreas are resistant to nitrogen mustard (22, 27) and roentgen irradiation (28) in the concentrations used.

Since the time between first administration of the 3 agents and the performance of the experiments (from 3-5 days) is too short to allow regeneration of the protein constituent of choline acetylase, it is unlikely that the increased acetylcholine synthesis resulting from administration of nitrogen mustard, roentgen irradiation and ACTH was due to increased concentration of choline acetylase. It is more in keeping with a relative increase in concentration of enzyme activators over enzyme inhibitors secondary to the decrease of the mass of the above mentioned tissues.

Since in hypophysectomized animals action potential can be initiated (1) and some acetylcholine can be synthesized (2), it may be inferred that ACTH is not indispensable for the occurrence of these 2 phenomena. ACTH, however, maintains the



function of nerve at a physiological level by preventing easy fatigability and by increasing the ability of nerve tissue to synthesize acetylcholine. The effect of ACTH in improving the function of nerve is dependent only in part on the reduction of the mass of thymus and lymphoid tissue.

#### SUMMARY

Hypophysectomized rats exhibited a marked decrease of the ability of nerve to maintain the amplitude of action potential during repetitive stimulation, and a decrease of the ability of nerve tissue to synthesize acetylcholine. Roentgen irradiation and administration of nitrogen mustard increased acetylcholine synthesis in hypophysectomized rats about 30 per cent, and increased by about 30 per cent their ability to maintain the amplitude of action potential during repetitive stimulation. Administration of ACTH restored in hypophysectomized rat the acetylcholine synthesis and the ability to maintain the amplitude of action potential to normal. The effect of ACTH in improving the function of nerve is probably dependent only in part on the reduction of the mass of thymus (perhaps also the lymphoid tissue).

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# ACTION OF COBRA VENOM AND CARDIOTOXIN ON GASTROCNEMIUS-SCIATIC PREPARATION OF A FROG

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THE site of action of cobra venom in a nerve-muscle preparation of a frog has been investigated by various workers. Lauder, Brunton and Fayer (1, 2) first demonstrated the curare-like action of cobra venom in dogs; this was verified by Wall (3) and others (4-6). Aron (7) and Martin (8), however, did not observe similar action of cobra venom. The paralyzing action of cobra venom on nerves has been shown by Gautrelet and his co-workers (9, 10) and according to them its action could not be prevented by merely obstructing the lymph circulation. Epstein (11), however, observed complete loss of excitability of voluntary muscles brought about by cape cobra venom and Kellaway and Holden (12) demonstrated the direct action of venom on muscles. The multitude of views thus held by different workers suggests further investigation in this direction, and we propose to study and compare the respective effects of cobra venom and cardiotoxin (an active principle isolated from cobra venom (13)) on the gastrocnemius-sciatic preparation of a frog.

## METHOD

Gastrocnemius-sciatic preparations were made from frogs of average weight of 200 gm. A muscle trough was divided into two chambers by interposing a paraffin wall across the trough, having a small groove for the transit of the nerve across the wall. The muscle was alternately stimulated through its nerve and through the muscle itself. If contraction of the muscle is obtained by direct stimulation even after stimulation through the nerve fails to excite any contraction, one can conclude then that the muscle fibers are stimulated. The respective times required for complete paralysis of the neuro-muscular junction and the muscle fibers were recorded and given in table 1.

It has been observed that when the nerve of a gastrocnemius-sciatic preparation of a frog is immersed in venom solution and the muscle in Ringer's, then on stimulation, either directly or through the nerve, the muscle shows contraction. Even the conducting power of the nerve is not affected when exposed to a fairly concentrated solution of venom such as 1 in 200. When the solutions are interchanged, the muscle loses its ability to contract in 10 minutes, showing thereby

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that either it acts directly on the muscle fibers or on the neuro-muscular junction. This holds true even in the case of cardiotoxin. Experiments were performed in order to decide whether they act on the muscle or neuro-muscular junction or on both. The results are given in table 1.

It has been observed that when the muscle is stimulated through the nerve after having been kept immersed in the venom solution of a definite concentration, there is gradual shortening of the amplitude of its contraction until, after a certain period of time depending on the strength of the solution used, it ceased to contract altogether (table 1). With weak solutions it goes into partial contracture and

TABLE 1. ACTION OF COBRA VENOM AND CARDIOTOXIN ON FROG GASTROCNEMIUS-SCIATIC PREPARATION

NO.	MUSCLE-CHAMBER SOLUTION	NERVE-CHAMBER SOLUTION	CONC.	TIME REQUIRED FOR NO CONTRACTION OF MUSCLE WHEN STIMULATED:	
				(1) Indirectly	(2) Directly
				min.	min.
A. 1	Venom	Ringer	1:5,000	55	90
2				65	110
3				50	85
4				65	95
B. 1	Venom	Ringer	1:10,000	85	130
2				70	115
3				80	120
4				75	125
C. 1	Venom	Ringer	1:20,000	180	310
2				200	335
3				190	335
4				170	300
D. 1	Cardiotoxin	Ringer	1:10,000	80	90
2				90	95
3				70	80
E. 1	Cardiotoxin	Ringer	1:20,000	125	135
2				110	115
3				130	160
F. 1	Cardiotoxin	Ringer	1:40,000	185	200
2				195	200
3				175	185

with strong, to complete contracture (provided the time of immersion remains the same). It will be noticed also from table 1 that when the stimulation of its nerve fails to evoke any contraction the muscle still shows some contraction on direct stimulation. On longer immersion, however, the muscle ultimately loses its power of contractility showing thereby that the fibers have been paralyzed. With strong solutions the action is fairly rapid and the muscle soon loses its ability to contract, only the time difference is not so great. However, with weak solutions the action is slow and the time difference is more pronounced. In fact, in all cases the paralysis of the neuro-muscular junction is found to occur earlier.

Similar experiments with cardiotoxin, on the other hand, showed that the time required for no contraction, whether stimulated directly or through the nerve, was almost identical. As usual, the time required increased as the concentration of the solution in the muscle chamber decreased, and also, as in cobra venom, the magnitude of contraction depended upon cardiotoxin concentration.

#### DISCUSSION

Since the muscle responds when stimulated through the nerve (treated with cobra venom) and the contractility remains the same, it may be concluded that cobra venom or cardiotoxin has no paralyzing action on nerve. When the solutions are interchanged—that is, when the muscle is dipped into venom or cardiotoxin solution—it does not show any contraction, whether stimulated directly or indirectly, showing thereby the paralysis of the neuro-muscular junction or the muscle, or both. The difference in time required for no contraction when stimulated directly and through the nerve (table 1) suggests that cobra venom acts both on the neuro-muscular junction and on the muscle. This lack in difference of time factors in the case of cardiotoxin (table 1) perhaps indicates its action only on the muscle, leaving the neuro-muscular junction intact. As to the mechanism of this action, nothing is known yet.

#### SUMMARY

The conducting power of the nerve is not much affected and remains almost the same when exposed to a solution of cobra venom or cardiotoxin. Cobra venom acts both on the muscle as well as neuro-muscular junction, the paralysis of which occurs earlier and in a relatively less concentrated solution. Cardiotoxin acts directly only on the muscle, leaving the nerve and the neuro-muscular junction intact.

We are grateful to Dr. B. N. Ghosh of the Calcutta University for the keen interest taken by him during the progress of the work. Our best thanks are also due to Prof. E. M. K. Gelling of the Chicago University who has very kindly helped us in writing this manuscript.

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## EFFECT OF TEMPERATURE CHANGE ON ROUND-WINDOW RESPONSE IN THE HAMSTER<sup>1</sup>

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THE study of many physiological mechanisms has been advanced through the use of temperature as a variable. However, the literature on the auditory system records only a few experiments in which temperature was varied over an appreciable range. Although Adrian, Craik and Sturdy (1) obtained valuable results in cold-blooded vertebrates, Wever states in his recent book (2) that "a study of the effects of temperature upon the electric response [i.e. the cochlear potentials] is difficult to make in the living animal because of its ability of temperature regulation." In order to avoid these difficulties we selected a hibernating mammal for the exploratory experiments reported below.

The hamster normally hibernates when put in the cold, and a wide range of temperature may be considered 'physiological' for this animal. Under barbiturate anesthesia the animal's body temperature is readily manipulated. This anesthesia, however, presumably has little direct effect on the electrical potentials recorded from the round window.

The electrical response of the ear has customarily been described as consisting of two types of components: the aural microphonic generated in the cochlea and the action potentials in the auditory nerve. Among the readily available acoustic stimuli sharp clicks have certain advantages since it is relatively easy to identify the so-called microphonic and neural components in the electrical response to clicks as recorded from the round window. The object of the present experiment is to describe

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the changes that occur in the round-window response of the hamster as the animal's body temperature is varied.

#### METHOD

Adult golden hamsters<sup>2</sup> (*Mesocricetus auratus*) were anesthetized by means of Dial with Urethane (Ciba) at a dosage of 0.1 cc/100 gm. This dosage produces anesthesia for 6 to 8 hours. The animal was pinned to a dissecting board, ventral side up, and the tympanic bulla was exposed by dissecting from a point just medial and posterior to the posterior tip of the mandible. The surrounding tissue was held back by steel wire retractors. A plastic tube 9.5 cm. long and approximately 0.15 cm. in inner diameter was introduced into the external auditory meatus and tied so that the tip of the tube was near to, but not in contact with, the eardrum.

Acoustic clicks from square-wave electrical pulses of 0.1-msec. duration were generated by a Permoflux earphone (PDR-10) to which was attached the plastic tube leading to the external ear of the animal. The clicks were presented at a rate of about one per second, and they could be attenuated below a reference level (the output of the click generator is here 0 db). The average human threshold for these clicks is about -80 db. The polarity of the clicks is changed by a reversing key in the circuit; thus clicks could be produced having either an initial condensation in the external canal or an initial rarefaction.

The recording electrode was a long thin steel needle with or without a saline-soaked wick at the tip. The electrode was placed by means of a micromanipulator so that the tip was touching the niche of the round window. The relative amplitudes of the microphonic and neural components of the response were found to vary with different electrode placements along the length of the cochlea; the round-window location was always used in the studies reported here. The electrical ground to the animal was generally the most anterior steel retractor in muscle and skin surrounding the opening leading to the bulla. The cochlear potentials picked up by the electrode were amplified and were then led to a Dumont 247B cathode ray oscilloscope and recorded photographically.

The temperature of the hamster was recorded from a clinical thermometer placed well into the cheek pouch on the side opposite the ear under study. The temperature was varied by circulating cold or warm water through a pair of copper coils between which the animal was placed.

A single experiment consisted of changing the temperature of the animal from 30°C. to approximately 18°C. and back to 30°C. over a period of approximately 3 hours. In a typical experiment the change from 32°C. to 22°C. took about one hour, from 22°C. to 18°C. another hour, and the entire return from 18°C. to 32°C. was accomplished in one hour. The temperature control apparatus allowed only relatively crude manipulation of the temperature of the animals. During the cooling process, whenever the animal reached the desired temperature, photographs were immediately

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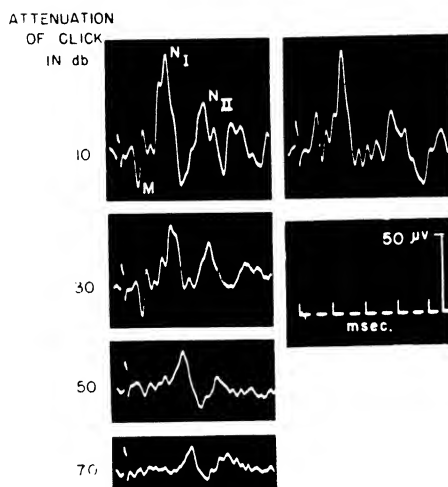
<sup>2</sup> The use of hamsters was suggested by Dr. Charles P. Lyman of the Department of Anatomy, Harvard Medical School; both he and Dr. Paul O. Chatfield (Department of Physiology, Harvard Medical School) have given valuable assistance in certain phases of this study.

taken of the response of the round window to clicks. If during the time photographs were being taken the temperature of the animal changed by a measurable amount, the mean temperature of the recording period was used.

This method of temperature control allowed us to explore the temperature range (as measured in the cheek pouch) from 18°C. to 39°C. Since cheek-pouch temperatures are known to correlate well with other evidences of metabolic activity in hamsters (3), they are preferable to rectal temperatures. It is not to be assumed, of course, that the cheek-pouch temperature is identical with the temperature of the cochlea. However, as a relative measure the temperature of the cheek pouch is adequate.

Five hamsters were used in the temperature explorations, and of these the last three supplied most of the data reported here. About a dozen others were used in

Fig. 1. ROUND-WINDOW RESPONSES TO CLICKS. *Left column* shows effect of decreasing intensity of click upon most prominent microphonic component (M) and neural components ( $N_I$  and  $N_{II}$ ) of round-window response. Record in *right column* shows effect of reversal of polarity of click: microphonic component reverses polarity, neurals do not. Downward excursion of microphonic represents rarefaction in external canal. Earliest regular upward deflection on all records (A) is artifact of 0.1-msec. duration which is simultaneous with electric square pulse delivered to earphone. (Temperature 30°C., hamster 3.)



preliminary studies. In all the animals studied the effect of temperature upon the round-window response is reproducible even in some minor details to an extent uncommon in electrophysiological research. The sampling problem therefore looms less large here than elsewhere.

The photographic records were read after they were enlarged ten times in a microfilm reader. The latencies of the various components (in the round-window response) were measured from the onset of the stimulus artifact to the *peak* of the excursion for that component. These 'latencies' all include an interval of about 0.3 msec., during which the sound is conducted through the tube from the earphone to the eardrum. The amplitude of the various components is taken as the distance in millimeters from the baseline to the point of maximum excursion. These readings were then converted into microvolts on the basis of calibration records. The data points on the graphs that follow represent median values computed from four or more separate pictures taken at a given temperature.



## CHARACTERISTICS OF ROUND-WINDOW RESPONSE AT CONSTANT TEMPERATURE

The typical round-window responses of the hamster at a constant temperature to clicks of varying intensity are illustrated in figure 1. The earliest excursion (M) to appear after the stimulus artifact is the most prominent microphonic component, and it is identified both by its latency and by the fact that it reverses in polarity when the polarity of the click is reversed. The next prominent excursion to appear will be called the first neural component ( $N_I$ ), and following this is the second neural component ( $N_{II}$ ). These neural components are always negative in polarity. The effects of attenuation of the click are also demonstrated in figure 1. As might be predicted, the less intense the stimulus, the smaller the amplitude of the microphonic and neural components. Another correlate of stimulus attenuation is the increased latency of the neural components (fig. 1, 2). There was considerable variability among animals in the relative sizes of the three components, M,  $N_I$  and  $N_{II}$ , of the round-window response. For a single preparation there is, however, sufficient stability, especially with respect to latency, to allow a meaningful study of the change of the round-window response as a function of temperature.

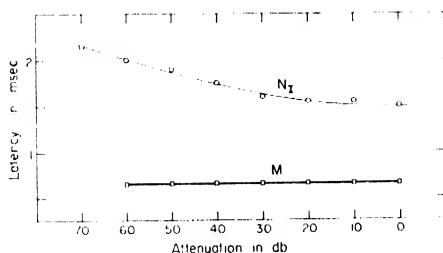


Fig. 2. LATENCY OF FIRST NEURAL COMPONENT and of most prominent microphonic component as function of intensity of click stimulus. Increase in latency of first neural component (circles) is appreciably less than increase measured as consequence of reduction of temperature from about 30°C. to 20°C. (fig. 3, 4). Note constancy of latency of M (squares).

## CHANGES IN ROUND-WINDOW RESPONSE AS A FUNCTION OF TEMPERATURE

**Microphonic.** As can be seen in figures 3, 4 and 5, the latency of the microphonic component M remains constant throughout the temperature range studied (18° to 39°C.). The greatest departure noted in a single record is less than 0.1 msec. The latency (which, it should be recalled, includes a sound-conduction time of about 0.3 msec.) always falls between 0.6 and 0.7 msec.

In contrast to its fixed latency, the amplitude of the microphonic falls significantly with decreasing temperature. For a temperature change of 30° to 18°C. the order of magnitude of this decrease is about one-third. Figures 3 and 6 illustrate the range of the phenomenon.

**First Neural Component.** The most dramatic change obtained in the round-window response as the temperature is reduced is the increase in latency of the first neural component (figs. 3-5). Latency changes of as much as 2.5 msec. were noted. These latency shifts were reasonably constant and predictable from animal to animal and for several experimental runs in the same animal. The latencies measured while the temperature was lowered were generally several tenths msec. longer than the corresponding latencies at the 'same' temperature during the warming part of the cycle. The duration of the first neural component increased appreciably with

decreasing temperatures. The measurements of response duration were, however, relatively unreliable. The size of the first neural component was markedly influenced by temperature (figs. 3, 4, 6). At about  $18^{\circ}\text{C}$ . the neural response was barely detectable at the click intensities used. Between  $18.5^{\circ}$  and  $30^{\circ}\text{C}$ . the amplitude of the first neural component increased by a factor of about 3 (at click intensities of  $-10$  and  $0$  db). At temperatures above about  $30^{\circ}\text{C}$ . there was little further increase in amplitude.

*Second Neural Component.* It is difficult to make precise and reliable measurements of  $N_{II}$ . However, it is clear that with lowered temperature the latency of the second neural component increases and that its amplitude decreases in a manner similar to the behavior of the first neural component (figs. 3, 4, 5).

#### DISCUSSION

The click responses from the round window of the hamster are strikingly similar to the click responses recorded from the round windows of other animals (cat, guinea-pig and bat) in this and other laboratories. The generality of this click response and the relative ease with which its microphonic and neural components can be separated and more or less independently manipulated by varying temperature seems worthy of note.

The preceding results raise several questions.

*Latency.* As the temperature is varied over an appreciable range, behavior with respect to latency differentiates clearly between the two components of the round-window response.

1) *Microphonic.* The time course of the events portrayed by microphonic potentials remains unchanged over a temperature range of  $20^{\circ}\text{C}$ . The microphonic, it is generally agreed, represents a preneural event, the origin of which is usually localized in the hair cells of Corti's organ. If this assumption is correct the evidence presented here states that peripheral to the hair cells the auditory system is time-wise insensitive to temperature.

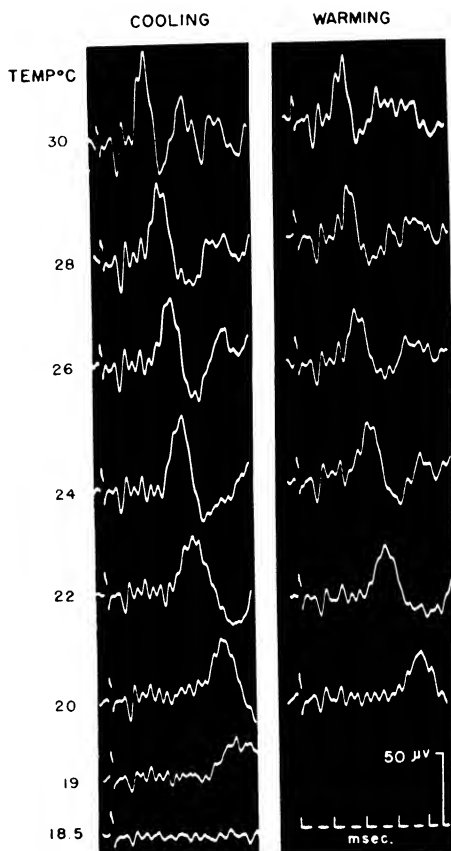


Fig. 3. ROUND-WINDOW RESPONSES during cycle of cooling and warming. Clicks at  $-10$  db. Neural components decrease in amplitude and increase in latency with cooling. During warming latencies shorten and amplitudes increase again. Microphonic component also decreases in amplitude with cooling; however its latency remains constant throughout.

2) *Neural components.* Before we can meaningfully discuss the spectacular increase in the latency of the neurals we have to make certain assumptions about the structures that give rise to these potentials. On the basis of experimental evidence we are inclined to ascribe  $N_I$  to the cell bodies of the spiral ganglion and to ascribe  $N_{II}$  to a more central structure, possibly the cochlear nucleus. These tentative assignments agree with the views recently expressed by Davis and his collaborators (4).

If the earlier neural component ( $N_I$ ) is indeed attributed to the cell bodies in the ganglion of Corti, then the delay introduced by a drop in temperature might occur at these cell bodies. There are, however, at least two loci more peripheral to the cell bodies where delays might equally well be introduced. These are the junction between the hair cell and the endings of the auditory nerve, and the segment of auditory nerve between the hair cell and the cell body.<sup>3</sup> There is no convincing evidence for either excluding or including any of these loci as sites where temperature might be acting, and, indeed, still others may well exist. Our attempts to press the analysis beyond this point have not therefore seemed profitable since, in the absence of substantially more information, too many dubious assumptions are required. The difficulties encountered in the attempt to explain the latency shift of the first neural component apply also to the explanation of the equally dramatic increase in the latency of the second neural component.

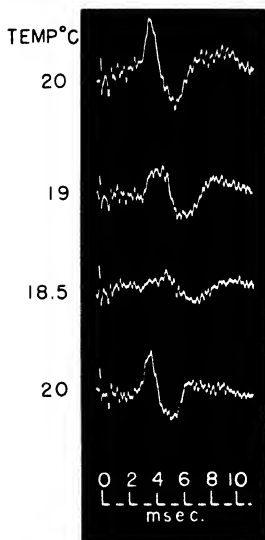


Fig. 4. MINIMUM POINT IN TEMPERATURE CYCLE of figure 3, to be read from top to bottom. Smallest neural response visually detectable occurred at 18.5°C. Recovery sets in as soon as temperature rises. Clicks at -10 db. Note different time scale.

<sup>3</sup> The endo-cochlear portion of the eighth nerve shows the axon (central to the cell body) to be relatively large, while the dendritic portion (peripheral to the cell body) is significantly smaller. Peripheral to the spiral lamina, as the dendrite approaches the region of the hair cell, its cross section is very small indeed, certainly less than a micron. We would, therefore, expect that the conduction velocity in these fibers would be small. This velocity would indeed have to drop to a value near 0.5 m/sec., if any appreciable part of the latency shift were to be explained in terms of the effect of temperature upon conduction rate.

Fig. 5. LATENCIES OF MICROPHONIC AND NEURAL COMPONENTS as function of temperature (clicks at -10 db). *Bottom graph* shows variation in latency of M and  $N_I$  during temperature cycle of figures 3 and 4. *Middle graph* shows data for more extended temperature range on same animal. Values for temperatures below 32°C. come from another temperature cycle, completed during same experimental day as bottom graph. Values for temperatures above 30°C. obtained the following day (symbols bear vertical lines). *Top graph* comes from another animal. Note how closely curves for M and  $N_I$  resemble analogous curves in lower graphs. Variation in latency of  $N_{II}$  could be measured in this animal. Curves, fitted by eye, are intended to illustrate main conclusions of graph: latency of M does not vary with temperature, latencies of  $N_I$  and  $N_{II}$  increase as temperature is decreased below about 30°C. Note that in general, for equal temperatures in a given cycle, latencies are shorter during warming than during cooling.

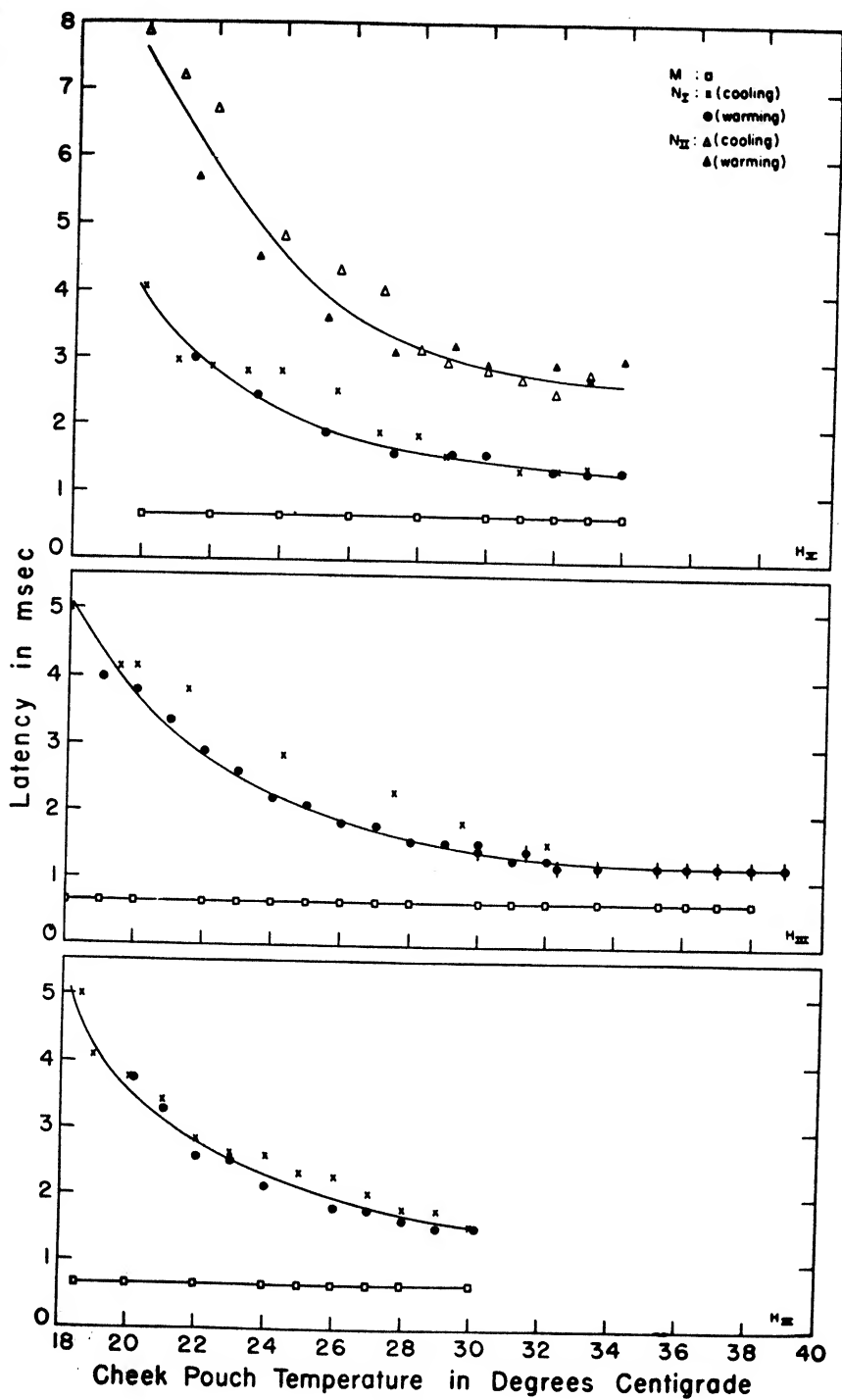


Fig. 5

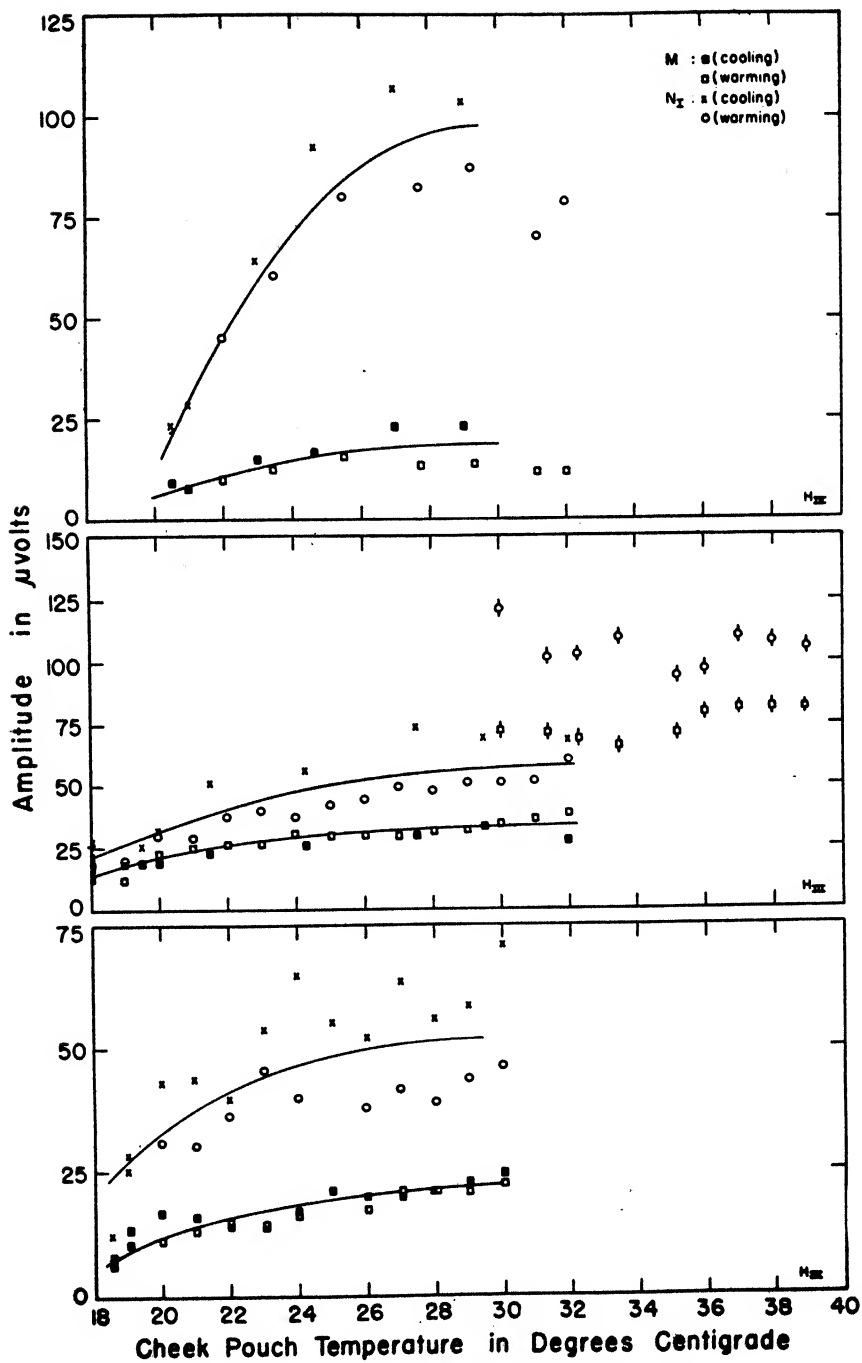


Fig. 6

*Amplitude.* The decrease in amplitude of the microphonic and both neural components with decrease of temperature has been demonstrated. If the temperature is lowered sufficiently the neural components decline faster in amplitude than the microphonic. A temperature is ultimately reached at which an acoustic click will elicit the microphonic components only. The temperature at which the neural components cease being observable depends upon the intensity of the stimulus: the more intense a stimulus the lower the temperature at which the stimulus will give rise to observable neural responses.

For both microphonic and neural components the principal effects of temperature were noted between 25° and 18°C. Why such effects should be restricted to a critical temperature range is off-hand not clear, and that they should be restricted to this particular one would scarcely be predicted from the data of Chatfield *et al.* (5).

*Variability.* As a comparison of figures 5 and 6 shows, there is considerably less variability with respect to latency than with respect to amplitude. A somewhat similar observation was made by Libet and Gerard (6).<sup>4</sup> Most of the small variability in latency can be reasonably attributed to difficulties in measuring it and to uncertainty regarding the actual temperature of the cochlea and of neighboring structures. As for the rather considerable variability in amplitude other factors have to be considered in addition to those just mentioned: contamination of neural components by microphonic potentials, position and contact of electrode, state of preparation etc. (cf. middle graphs, fig. 5, 6).

Another aspect of variability is *reversibility*; we define reversibility here as our ability to obtain equal values for the dependent variables, latency and amplitude, for equal values of the manipulable variable, temperature, during the course of a particular temperature cycle. Figures 5 and 6 demonstrate again that the phenomena are clearly more nearly reversible with respect to latency than with respect to amplitude.

As can be seen from figure 5, at equal temperatures latencies are shorter during warming than during cooling. This could be accounted for by assuming a lag of the cheek-pouch temperature with respect to the 'cochlear temperature.' During the cooling the cochlea would actually be cooler than the thermometer indicates, while during warming the cochlear temperature would be higher than that read from the cheek pouch. On the basis of this explanation we should predict that amplitudes during warming should exceed those obtained at the same temperature during cooling. Our evidence (fig. 6) shows, however, just the opposite to be true, for clearly

<sup>4</sup>They report more variability in their  $Q_{10}$  for amplitude than in the  $Q_{10}$  for frequency in their paper, "Potential Waves from the Isolated Frog Brain."

Fig. 6. VARIATION IN AMPLITUDE of microphonic and neural components as function of temperature. *Bottom and middle graphs* contain amplitude data that go with latency data in corresponding parts of figure 5. In *middle graph* symbols bearing vertical lines illustrate amount of variability in amplitude encountered in same animal on consecutive experimental days with same apparent electrode placement. Cooling phase for this cycle is absent since animal died when temperature of 40.5°C. was reached. *Top graph* represents similar run in another animal. In general amplitudes of both microphonic and neural components decrease with cooling. There is much greater variability in amplitude data than in latency data. Note how, in general, for equal temperatures, amplitudes are smaller during warming than during cooling.

the amplitudes of all components during warming are smaller than they were at the 'same' temperature during cooling. This is a puzzling difference in behavior with respect to amplitude and to latency about which we can speculate but which we feel unable to explain.

One factor presumably important in considerations of reversibility is the rate at which the temperature of a preparation is changed. The time required to attain equilibrium at a given temperature has not been carefully explored in these experiments, but one striking example of its importance may be cited. An animal cooled 'slowly' to 18°C. showed a fair-sized neural response at that temperature; one hour later no response could be seen, and it reappeared only after warming to 19°C. was accomplished.

Latency and amplitude are of course not the only temperature-dependent variables that can be examined. As previously indicated, there is a noticeable, though not too easily measurable, increase in the duration of the neural components. As a next step we might consider how the area (voltage  $\times$  time) of the first neural component varies with temperature. Almost by inspection we can see that over a relatively wide temperature range this area would remain fairly constant since the decrease in amplitude is compensated by increased duration. Were we to interpret the neural components as a distribution of discharges from a certain number of neural units, we could say that a lowering of the temperature makes the discharges at first less synchronous. If the temperature drops, however, below a certain critical value, we are unable to observe any kind of synchronized activity from an appreciable number of neural units in response to a stimulus of a given strength.

#### SUMMARY AND CONCLUSIONS

Our evidence on the differential effect of temperature upon the various components of the round-window response supports the opinion of most workers in the field: different processes in the end organ are responsible for the microphonic and neural components of the round-window response. Our findings on the effects of temperature upon latency, duration and amplitude are also in agreement with those reported by numerous workers (among others, Rosenbluth *et al.*, 7) though contradictory evidence, especially on the relation of amplitude to temperature, is by no means lacking (see for instance Schoepfle and Erlanger, 8). The following results can however be considered as established, no matter what interpretation is put upon the explanation of the effects.

The round-window response of the hamster cochlea to acoustic clicks is described. The microphonic and neural components identified are similar in all important aspects to those found in the cat and the guinea-pig. When the body temperature of the hamster under Dial-Urethane anesthesia is varied between 18°C and 39°C., systematic changes in the electrical response of the cochlea are observed. These changes are essentially reversible. The amplitude of both microphonic and neural components is decreased by cooling below 30°C. The neural component declines more rapidly than the microphonic, and it disappears at a temperature where the microphonic is still observable. The greater the stimulus intensity, the lower the temperature at which it fails to evoke the neural component. The latency

of the most prominent microphonic component remains constant and is thus independent of temperature over the range investigated. There is a striking progressive increase in both latency and duration of the neural components as the temperature is decreased below 30°C.

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# GLUCOSE SPACE OF THE BODY<sup>1</sup>

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**F**EW measurements of the glucose space of the body have been made. One method of measuring the space or volume of the body in which a substance is dissolved involves the injection of a known amount of the substance and measurement of the dilution during the circulation of the blood and possible diffusion into extracellular or cell fluids. With glucose it is not possible in the intact animal to use the conventional method. The rise in the blood sugar consequent upon the injection of glucose sets up mechanisms, particularly in the liver, which take glucose from the circulation and change it to other forms. The method may be used in the liverless animal although even here one cannot be sure that the action of other organs may not similarly invalidate the determination as a result of the increased blood sugar level. Radioactive glucose labeled with carbon-14 offers a means of avoiding this complication; one may inject a minute amount of highly potent material which would have a negligible effect on the blood sugar level and the dilution of it can be accurately determined.

In carrying out this work we were chiefly concerned with the eviscerated animal. This is because of the usefulness of this preparation in studies of intermediary metabolism and because of the large amount of work done with it in the past. In such work it is often necessary to calculate from the change in blood sugar concentration the change in glucose content of the body. In the past workers have had to assume a value for the glucose space to use such calculations (1, 2).

In addition we have carried out estimations of the glucose space in intact animals. Such a determination has some value in experimental work. However, it cannot be measured with the accuracy with which it may be in the eviscerated animal. This is chiefly because of the complicating factor of the liver which would add an unknown amount of glucose to the body during the time, between injection and sampling, needed for mixing. To some extent a similar complication may result from the presence of the intestinal tract with its contents although this can be minimized by a long preliminary fast.

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## METHODS

*Treatment of Experimental Animals.* Rabbits were used. In all cases the animals were fasted at least 4 days prior to the determination. For evisceration we used the technique described in an earlier paper (3). In some animals the kidneys were removed in order to obviate any possible formation of glucose by them. After recovery from the operation and after we were assured that the animal was in good condition, 60 mg. of radioactive glucose were injected intravenously. In the intact animals 10 mg. of highly potent radioactive glucose were injected. A sample of blood was taken approximately 30 minutes thereafter in order to determine the degree of dilution by the body fluids. This time has been shown to be adequate for substances that are distributed within the extracellular fluid (4). If the glucose space extended beyond this limit it might require a longer mixing time for equilibrium. This question was investigated in some animals in which repeated samples of blood were taken at various intervals. The results are given later under 'Speed of Mixing.' A 15-ml. sample of blood was drawn; clotting was prevented with a small amount of heparin and the plasma was separated by centrifugation. Estimations on this plasma were then carried out for the glucose, amount of radioactivity per ml. and the specific activity of the glucose.

*Plasma Glucose Determination.* The plasma glucose determination was carried out by a combination of micro fermentation with Fleischmann's Baker's yeast as described by Reinecke (5) and the ferricyanide-ceric-sulfate macro procedure of Miller and Van Slyke (6). The plasma was deproteinized with cadmium hydroxide and the excess cadmium removed with barium carbonate. The difference between the glucose equivalent of the non-fermentable reducing substance and the conventional plasma sugar value was taken as the glucose equivalent of the fermentable reducing substances in the blood.

*Plasma Radioactivity.* For the determination of the total radioactivity (other than that of  $\text{CO}_2$ ) per unit volume of plasma, 1 ml. of plasma was mixed with 1 ml. of 0.5 M phosphate buffer. This mixture was frozen, lyophilized and weighed. The  $\text{BaC}^{14}\text{O}_3$  obtained from dry combustion was measured for radioactivity.

*Plasma Glucose Specific Activity.* The specific activity of the plasma glucose was obtained from the  $\text{CO}_2$  produced by the fermentation with Fleischmann's Baker's yeast of 1 ml. of plasma and 60 mg. of glucose added as carrier. The  $\text{CO}_2$  was precipitated as  $\text{BaCO}_3$ .

*Measurement of  $\text{C}^{14}$  Radioactivity.* All radioactive determinations were carried out on  $\text{BaCO}_3$  (7) obtained by either fermentation or dry combustion. In all cases the samples were of infinite thickness.

*$\text{C}^{14}$  Labeled Glucose.* The radioactive glucose was uniformly labeled and was obtained by biosynthesis (8).

## RESULTS

*Speed of Mixing.* In order to determine the time needed for equilibrium to be established between injected glucose and the body glucose, samples of blood were taken at various times after injection of the radioactive glucose. These animals had been nephrectomized and eviscerated. The specific activities of the glucose of these plasmas were determined by the method described above. If mixing with the extra-

vascular space were incomplete in the first sample the glucose specific activity would be higher than that of subsequent samples. Any utilization of glucose by the tissues between the times of sampling would not invalidate the conclusions drawn from these results, since the labeled and the plain glucose would be used in proportion to the relative concentrations in the body fluids and the glucose specific activity would not be changed. The results for *rabbits* 13, 16, 17 and 26 are shown in table 1. It appears that the radioactive glucose equilibrates within 20 minutes and that the body glucose which dilutes it is mixed very readily with it.

In two animals (table 1—*rabbits* 13 and 26) we took samples 3 hours apart in order to see whether there might be a slow process of dilution by some body glucose that would be more inaccessible for mixing with ordinary circulating glucose. Intracellular glucose might be considered to act in this way. We see here that even after 3 hours there is no evidence of a slow dilution of the labeled glucose. The low level of the plasma glucose in the last sample makes this test particularly sensitive, since

TABLE 1. COMPLETE MIXING OF INJECTED RADIOACTIVE GLUCOSE WITH BODY GLUCOSE IN 20 MINUTES AND NO FURTHER DILUTION THEREAFTER

ANIMAL NO.	TIME BETWEEN INJECTION AND SAMPLE	PLASMA GLUCOSE	SPECIFIC ACTIVITY OF PLASMA GLUCOSE <sup>1</sup>
	min.	mg. %	
13	84	126	10,300
	265	22	12,600
16	30	198	8,150
	60	157	7,350
17	32	166	6,600
	65	134	7,500
26	22	138	2,850
	43	128	2,860
	200	42	3,120

<sup>1</sup> Counts per mg. of total carbon per minute.

the mixing of a relatively small amount of unlabeled glucose with the circulating glucose at this time would have given a definite decrease in its specific activity.

*Calculation of Glucose Space.* This was calculated by two independent methods. One of these was based on the concentration of active material in the plasma after mixing. The degree of dilution of the glucose specific activity was used to make the other calculation. In both instances a correction had to be made for the glucose utilization of the tissues between the time of injection and that of the sampling. This can readily be done since in the 4-day fast preparations the rate of fall of the blood sugar is fairly uniform (9). In this series we found the blood sugar level to drop at the rate of 30 mg. per cent per hour or half a mg. per cent per minute. We assumed that the labeled glucose would be utilized at the same relative rate as the total glucose. The two methods of calculation are illustrated from the following actual data.

R 26—weight after evisceration and nephrectomy was 2.11 kg.; at 11:10 A.M. the animal was injected with 69 mg. radioactive glucose (specific activity = 35,600 ∴ each mg. glucose = 14,200 cts/mg/min. At 11:30 A.M. a blood sample was taken.

Plasma sugar = 120 mg. per cent. At 11:10 A.M. (just after injection) the blood sugar was assumed to have been 130 mg. per cent (120 mg. per cent + 0.5 mg. per cent per min.  $\times$  20 min.). Each ml. plasma had 1,420 cts/min. by dry combustion. Specific activity of plasma glucose was 3,320 by fermentation.

*First Method of Calculation.* Glucose concentration was assumed to have dropped from 130 to 120 mg. per cent in the 20 minutes between injection and sampling. This represented a utilization of  $1/12$  circulating glucose and we assumed the same utilization of the injected radioactive glucose ( $1/12$  of 69 = 5.8). Therefore the amount of labeled glucose in the body at the time of sampling was 63.2 mg. which would have a total count of 895,000 cts/min. Since each ml. of plasma had a count of 1,420 cts/min. the glucose must have been distributed in a space of  $897,000/1,420$  or 525 ml. This would represent 24.9 per cent body weight.

*Second Method of Calculation.* The specific activity of injected glucose is 10.7 times greater (35,600/3,320) than that of the plasma glucose, and we may assume that the injected glucose was diluted by unlabeled glucose to this extent. Therefore

TABLE 2. RESULTS OF DETERMINATION OF GLUCOSE SPACE IN VARIOUS TYPES OF PREPARATIONS<sup>1</sup>

PREPARATION	ANIMAL NO.	METHOD #1	METHOD #2
Evisceration plus nephrectomy	16	24.9	27.0
	17	26.3	30.1
	26	27.5	28.2
Evisceration	23	29.5	30.3
	29	23	23
	30	21.6	26
Intact	28	31.8	
	31	30	31.5

<sup>1</sup> Figures are expressed as percentage of body weight.

immediately after injection of the radioactive glucose there were  $10.7 \times 69$  or 740 mg. glucose in the body. The plasma sugar level at this time was assumed to have been 130 mg. per cent.

Glucose space =  $\frac{\text{total glucose in body}}{\text{conc./ml. of plasma}} = \frac{740}{130} = 570 \text{ ml.} = 27 \text{ per cent body weight.}$  The results of these experiments are summarized in table 2.

#### DISCUSSION

Our figures for the glucose space are similar to those for the thiocyanate space in rabbits (10). This would suggest that the space we measured was identical with the extracellular compartment.

It is of interest to compare the glucose space of our animals with the galactose space as reported by Levine *et al.* (11). These workers used eviscerated dogs and found this space to be 46 per cent of the body weight. This figure indicates that galactose must pass to a considerable degree into the intracellular compartment. Equilibrium is only established after two hours. The degree of dilution of the galactose in the first half-hour after injection is similar to the dilution of glucose as found by

us. This would suggest that the later and slower phase of equilibration of galactose is due to passage into the cell, the rapid dilution being due to distribution within the extracellular phase. Glucose equilibrates rapidly in the extracellular phase and thereafter there is no further dilution of the labeled glucose; it is not further diluted by any intracellular glucose as is indicated by the constancy of the specific activity up to 3 hours or more. Undoubtedly it enters the cell but must do so by an irreversible process. Otherwise in our experiments unlabeled glucose from within the cells would have slowly diluted the radioactive glucose in the extracellular compartment. This disappearance of glucose parallels its utilization so that we may infer that once glucose enters the intracellular compartment, it is involved in an irreversible process which leads to a change of it to some other form such as glucose, phosphates, other intermediates,  $\text{CO}_2$ , glycogen, fat etc.

#### SUMMARY

The glucose space of the rabbit has been measured by the injection of small quantities of carbon 14-labeled radioactive glucose. Dilution of the injected glucose is complete in 20 minutes and no further dilution takes place in 3 hours or more. Two independent methods of calculation—one based on the concentration of radioactive material in the plasma after mixing and the other making use of the injected glucose specific activity, gave almost identical results. The figures for the glucose space are the same as those for the thiocyanate space of the rabbit and little more than half of those reported for the galactose space of the dog. As a percentage of the body weight the glucose space after evisceration averaged 26 per cent, after evisceration and nephrectomy, 27 per cent and in the intact animal, 31 per cent of body weight.

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# ELECTROTONIC POTENTIALS ELICITED BY THRESHOLD STIMULI FROM SHEATH-FREE NERVE AT DIFFERENT TEMPERATURES

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THE experiments to be described represent a further attempt to investigate the physical phenomena which determine the kinetics of excitation in nerve and muscle fibers. In a previous investigation by Schoepfle and Susman (1) rectangular current stimuli of various durations were applied to frog sciatic nerves from which the perineural sheath had been dissected. Initiation of propagated responses in the most irritable fibers appeared to depend upon attainment of a critical electrotonic potential at the cathode without regard to time rate of the potential change. The potentials recorded at the extrapolar edge of the stimulating cathode were assumed directly proportional to the voltage changes induced across a local portion of the distributed membrane capacity in accordance with the view that axoplasmic and external fluid currents are strictly parallel in extrapolar regions (2-4).

However, further investigation with more adequate elimination of extraneous transients has shown that additional refinements in experimental technique are necessary. The records in figure 1 of electrotonic potentials at extrapolar, midpolar and intrapolar edges of the cathode exhibit components which, in their deviation from rectangularity as time functions, coincide rather well. Such a component of the voltage time curve recorded at the extrapolar edge of the cathode constitutes potential changes which are most reasonably proportional to voltage changes across the distributed membrane capacity at that particular region. Hence it may be concluded that changes in membrane voltage follow very nearly the same time course at any point in the immediate region underlying the comparatively narrow cathode. Records from the extrapolar edge may thus be taken as representative of the whole region.

If local membrane voltage attains a critical value at threshold across the capacity it should decline on break of the stimulus at a rate which is relatively but not completely independent of the rate at which the distributed capacity was charged. One might therefore expect very nearly identical time functions for membrane voltage decline in each instance of threshold rectangular current stimulation. However, if some component of the potential record prior to break is due to flow of external fluid current in a direction not parallel to the long axis of the fiber it would most certainly appear to increase with the applied voltage of the stimulus source. On break of

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stimulus this current would disappear very quickly and the subsequent potential record would represent only voltage changes across the underlying membrane capacity.

In the present series of experiments electrotonic potentials elicited by threshold rectangular currents of various durations were recorded at different temperatures. Brief conditioning and testing shocks separated by various intervals were also employed to raise electrotonic potentials to a threshold level.

#### EXPERIMENTAL RESULTS

The voltage-time records of figure 2 are those of threshold electrotonic potentials recorded at the extrapolar edge of the stimulating cathode. Responses of the most irritable fibers just fail to appear. *Record A* was produced by a stimulus of 667 per cent rheobasic value. This particular curve was translated as a whole along the time

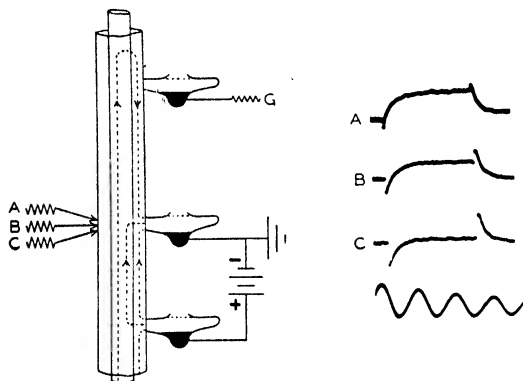


Fig. 1. NERVE FIBER surrounded by a thin external fluid film. Dotted lines indicate direction of current flow. Battery indicates a source of rectangular current stimuli. In a whole frog sciatic, stripped of perineural sheath, a knife-edge silver plate is placed at levels corresponding to regions A, B, and C in order to record the electrotonic potentials at threshold indicated in the oscillographic records at the right. Time calibration represents 1000 cycles. Distance between leads C and G is at least 15 mm.

axis until its declining phase coincided with that of a potential elicited by a rheobasic stimulus. The superimposed records are shown in *B*. It should be noted, however, that in order to obtain the coincidence, the stimulus break for the brief stimulus must occur a tenth of a msec. or so prior to that for the rheobasic stimulus.

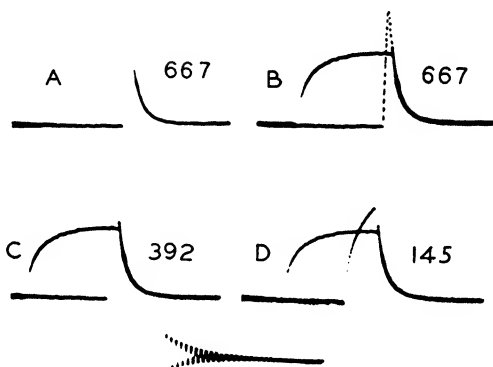
The apparent discrepancy most reasonably involves the overshoot of potential which must follow break of the stimulus prior to attainment of a steady state voltage across a portion of the distributed membrane capacity (3). In this instance the rate of voltage change with respect to time is relatively independent of the previous history of the system only after a tenth of a msec. or two have elapsed. The voltage must overshoot to the critical threshold value before it can decline at a rate relatively independent of that at which the capacitative system had been charged. However, on break of a rheobasic stimulus the voltage declines at a rate independent of previous history in so far as it is a function only of the impedance and steady-state rheobasic voltage at instant of stimulus break. The later portions of the two voltage time curves can thus appear identical in configuration without exactly coinciding in time.

The actual overshoot at stimulus break is obscured, in all probability, by an additional voltage component at the extrapolar edge of the cathode due to non-

longitudinal flow of current in the external fluid film. The superimposed records of threshold potentials shown in figure 2 *D* are relevant in this connection.

It will be noted that the electrotonic potential associated with the brief stimulus rises to a height greater than that for the rheobasic and yet follows an identical time course throughout most of the decline subsequent to stimulus break. Most reasonably, this implies that part of the potential record is due to potential differences in external fluid and at nerve electrode junction due to flow of current in a direction not parallel to the long axis of the fiber. Such a component vanishes quickly on break of the stimulus leaving manifest only the slower voltage decline across the distributed capacity. Hence it would seem that analysis of the curves subsequent to break of the stimulus provides the most reliable means of investigating the membrane potential proper in its relation to excitation.

Fig. 2. ELECTROTONIC POTENTIALS elicited by threshold rectangular current stimuli of various duration. Voltages of the external fluid with respect to a remote lead were recorded by a knife-edge silver plate at the extrapolar edge of the stimulating cathode as indicated in figure 1. The whole frog sciatic nerve is stripped of perineural sheath. Effective width of the calomel half cell stimulating cathode in contact with the nerve is 1.2 mm. Responses of the most irritable fibers just fail to appear. Record A, elicited by a 667 per cent rheobasic stimulus, was translated along the time axis to the point at which its declining phase coincided with that of a rheobasic stimulus. The superimposed tracings constitute record B. Records C and D consist of similar superimpositions. The numbers adjacent to each set of records indicate intensity of applied stimulus for the briefer shock in terms of per cent rheobase. Only dotted portions of the records are retouched. Time of stimulus make for the briefer shock is indicated by break in baseline. Time calibration represents 10,000 cycles. Temperature is 23°C.



Records of figure 2 were obtained at 24°C. Lowering of the temperature to 12°C. produced only slight changes in the time parameters of the potentials shown in figure 3. Chronaxie increased from 0.36 to 0.50 msec. In five instances a change in temperature produced no appreciable effect on the time parameters of either excitation or electrotonic potential as indicated by the example described in figures 4 and 5. No definite reason can be ascribed to the difference in result from one experiment to another. However, it can be stated with reasonable assurance that temperature has very little effect on the time parameters of either excitation or electrotonic potential in frog nerve stripped of perineural sheath. At any rate there remains the close correspondence between excitability and electrotonic potential when cooling does exert retarding effects.

In further experiments a double shock method was employed to elicit threshold electrotonic potentials. Brief subthreshold conditioning shocks were followed at various intervals by test shocks of just sufficient intensity to raise the most irritable



fibers to threshold. Configuration of the shock is shown in figure 7. Electrotonic potentials at the extrapolar edge of the cathode initiated by single shock stimuli of 98 per cent and 100 per cent threshold intensity are shown in figure 6, *A* and *B*. Responses of the most irritable fibers appear quite obviously in *B* but not in *A*. Superimposition of the records shown in *C* indicates that the ordinates of *A* are, for practical purposes, those of a threshold electrotonic potential.

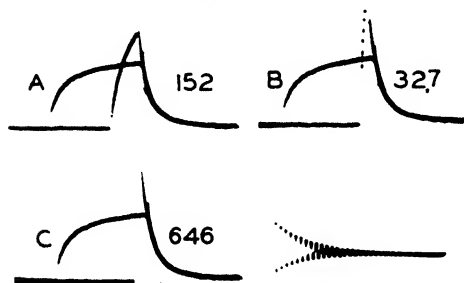


Fig. 3. THRESHOLD ELECTROTONIC POTENTIALS obtained as indicated in figure 2. Same nerve at 12°C. Time calibration, 10,000 cycles.

The voltage time curve *A* of figure 6 can be superimposed on electrotonic potential records obtained by double shock stimulation as indicated in figure 6 *D*, *E*, *F* and *G*. In this instance superimposition is merely contingent on translation of the single shock record along the time axis to the point at which its initial rise is coincident with that of the test shock. The curves obviously coincide throughout most of the descending phase. Here again records at peak fail to coincide presumably because of external fluid voltage components due to non-longitudinal current flow. However,

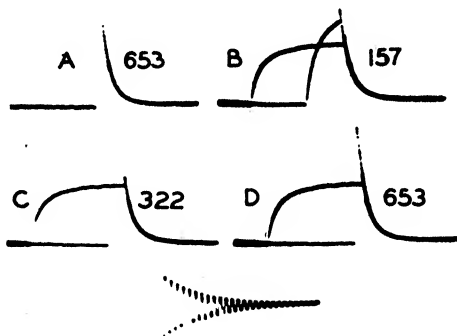


Fig. 4. THRESHOLD ELECTROTONIC POTENTIALS obtained as indicated in figure 2, but from another preparation at 23°C. Effective width of cathode in contact with nerve is about 2 mm. Time calibration, 10,000 cycles.

the subsequent portions of the voltage decline curves indicate identical changes in potential across the underlying capacitative mechanism.

Additional evidence is thus obtained in support of the view that initiation of a propagated response is dependent upon attainment of a critical voltage difference across a localized region of the distributed capacity without regard to the voltage-time function characterizing the charging process. This, of course, can apply only within the limits such that accommodation can be neglected.

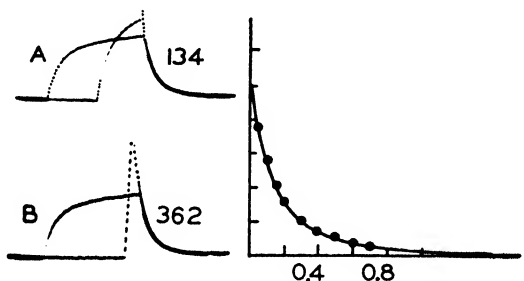
Records in figure 7 indicate that in this particular nerve, cooling is without effect on the time parameters of either excitation or electrotonic potential.

#### DISCUSSION

The perineural sheath was dissected off the nerve in order to eliminate any possible extraneous capacity or impedance of any sort that might tend to obscure the basic relation between local electrotonic potential and excitation (1). It is relevant to note that Schmitt and Stewart (5) have emphasized the necessity of sheath removal in order to evaluate properly impedance components of the membrane proper. Hence removal of the sheath most certainly allows the system to approximate more nearly one in which a single fiber is surrounded by a thin film of conducting fluid. Only in such a system can membrane voltage changes be clearly reflected in potentials recorded from the surface.

The question still remains as to whether the multifiber preparation employed is capable of disclosing the mechanism of excitation in a single fiber. The impedances

Fig. 5. THRESHOLD ELECTROTONIC POTENTIALS at 9° C. from the same preparation as that of figure 4. Curves at right indicate voltage following break of stimulus plotted as per cent normal rheobasic voltage. Continuous curve at 23°C.; solid circles at 9°C. Effective width of cathode, 2 mm. Time scale given in msec.



of most of the fibers should be nearly identical if the multifiber system is to be adequate. Now if the individual fiber impedances vary widely one would not expect to find that threshold of the most irritable fibers is attained when the overall electrotonic potential rises to a unique critical value. However, if potential and excitability appear to be functionally related as indicated in the data already presented it would seem that the multifiber system is a quite adequate one, composed for the most part of nearly identical fibers as far as impedances are concerned. In this connection Blair and Erlanger (6) report very nearly identical chronaxies for fibers varying in diameter through a range that probably includes most of the A fibers. There is consistent evidence, then, to indicate that most of the fibers contributing appreciably to the electrotonic potentials have impedances which are very nearly identical.

The technique employed of cooling the nerve was designed to test the functional relationship between excitability and electrotonic potential under conditions which presumably would retard time rates of both phenomena. Strikingly enough the retarding effects of cooling were vanishingly small in many instances and certainly slight when present. The low temperature coefficient indicated favors a view that physical rather than chemical processes are involved in the sequence of events leading to charging of the membrane capacity. The slightly more pronounced temperature

effects obtained by Blair (7) in chronaxie may have involved presence of the sheath as well as a metal stimulating electrode system.

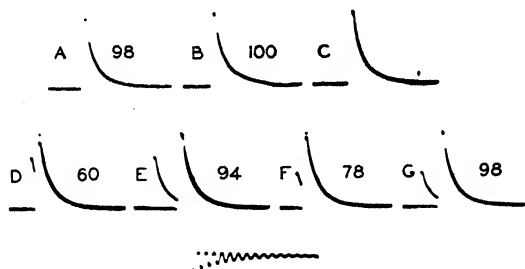


Fig. 6. ELECTROTONIC POTENTIAL at the extrapolar edge of the stimulating cathode elicited by shocks whose time course is shown in figure 7. Voltages of *A* were initiated by a shock 98 per cent as strong as that giving rise to those of *B*. Curve *C* is a superimposition of records *A* and *B*. Responses of the most irritable fibers are evident in *B* and *C*. Consecutive conditioning and testing shocks give rise to potential records *D* through *E*, upon which are superimposed in each case the curve of record *A*. Curve *A* was translated along the time axis until its time of onset was coincident with that of the test shock. Intensity of test shock in terms of per cent single shock threshold is indicated by the numbers adjacent to each figure. Effective width of cathode in contact with nerve is 1.2 mm. Temperature is 23°C. Time calibration, 10,000 cycles.

#### SUMMARY

Electrotonic potentials induced by threshold rectangular current stimuli of various durations were recorded at the extrapolar edge of the stimulating cathode in frog sciatic nerve stripped of perineural sheath. Curves of potential decline subsequent to break of the stimulus exhibit an identical configuration with respect to

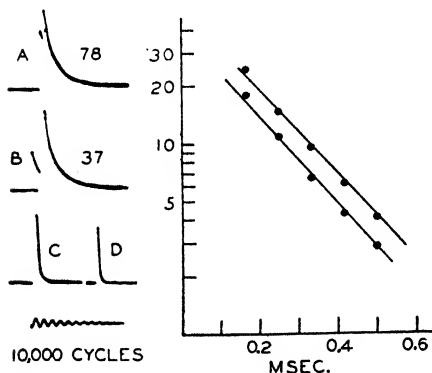


Fig. 7. OSCILLOGRAPHIC RECORDS *A* AND *B* were obtained as in figure 6 but at 9°C. from the same nerve. Curves at right are semilogarithmic plots of potential records obtained at 23°C. (upper curve) and 9°C. (lower curve). Effective cathode width is 1.2 mm. Record *C* is an electrotonic potential at the extrapolar edge of the stimulating cathode from a nerve previously killed by strong direct current. Record *D*, describing the time course of the applied stimulus, is obtained by leading directly to the amplifier.

time after a tenth of a msec. has elapsed. On relative translation of the curves along the time axis it is found that the curves coincide if time of stimulus break for the brief stimuli occurs a tenth of a msec. or so prior to that for the rheobasic stimuli.

These data are consistent with the proposal that attainment of a critical membrane voltage is sufficient for initiation of a propagated response. The apparent

difficulties involved in obtaining coincidence of the voltage-time curves were resolved on noting that for very brief stimuli, steady state voltage is not attained across the distributed membrane capacity. Voltage must overshoot to a critical threshold value over a local cathodal region after stimulus break before it can subside at the rate characteristic of the rheobasic decline, whose time function is relatively independent of the previous history of the system.

Electrotonic potentials elicited by a brief subthreshold conditioning shock and a subsequent threshold testing shock eventually decline along an identical time course in each instance regardless of conditioning shock intensity or interval between stimuli. These data provide further evidence which strongly supports the conclusion that local attainment of a critically outwardly directed potential difference across the distributed membrane capacity is sufficient to initiate a propagated response. Time limits are such that accommodation can be neglected.

Cooling of the preparation through  $10^{\circ}$  or  $15^{\circ}$  has little or no effect on the time parameters of either electrotonic potential or excitation. When retarding effects are appreciable, as they are unaccountably in some experiments, there remains the close functional relation between electrotonic potential and excitability.

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# ION BINDING IN MUSCLE HOMOGENATES

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IN VARIOUS tissues of vertebrates, potassium is in higher concentration within the cellular units than in the extracellular medium. The reverse situation is found for sodium and for chloride. Presumably this selective distribution of elements is general for a wide variety of cells, plant and animal. In many instances it has been shown further that alteration of the environmental ionic composition will cause alterations in the concentrations of the elements inside cells, with, in some cases at least, recovery of original conditions upon return of the cells to the original environment. Such an exchange between the two phases has been shown clearly during various phases of activity and return to rest of muscle and nerve tissues.

Recent investigations with radioactive isotopes of alkaline and alkaline-earth elements have shown clearly a fairly rapid exchange between cell and environment (1, 2). Further analysis of the facts have led to the conclusion that there should be, presumably at or near the surface of the cell, a selective sodium binding condition which could serve both as an exchange and as a transport mechanism (1). If an exchange-transport system for sodium did exist, then the distributions of non-transported potassium and chloride would follow from the usual Donnan relationships. The details of the passive distribution of K and Cl have been admirably worked out by Conway and his co-workers (3) for muscle tissue.

While the hypothesis of a specific sodium complex (or state of affairs) is strongly indicated by the evidence relating to ion distribution and movements between cells and tissues (1, 4), the whole idea suffers from the lack of direct evidence of any specific binding of either potassium or sodium. Various reports have claimed a potassium combination. In most instances, however, the evidence presented has not been extensive enough to show that the combination reported is specific for any one ion.

For preliminary investigations on selective ion binding, the distribution of Na and K between supernatant and residue of a centrifuged muscle homogenate has been studied. This procedure was selected because it involved no artificial structural and chemical additions to the system such as might complicate studies of ultrafiltration or dialysis. It is known that cellophane, among other cellulose derivatives, can act as a selective ion adsorbent.

It must, of course, be recognized that there may be no necessary correspondence between ion distributions in a muscle homogenate and the ion distributions of the original intact tissue. Nevertheless, properly used, the method of studying distribution of materials in particulate and non-particulate material in tissue homogenates has given encouraging results.

The type of ion binding of interest in the present work is specific retention of one ion in preference to another. Any phase of the muscle homogenate that includes

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base binding groups will carry cations with it and hence could be said to bind the ions. However, to fulfill the physiological requirements of cation balance between cells and environment, systems must be present which selectively bind one element or the other, presumably sodium.

#### MATERIALS AND METHODS

Thigh muscles of grass frogs were excised, cooled and rapidly homogenized with 0.2 M sucrose<sup>1</sup> at 0° to 2°C. to a final volume such that each ml. of homogenate contained about 0.07 gm. of tissue. Homogenizing was carried out using a variation of the Waring Blender mechanism. Stainless steel blades, one rotating and one fixed, were arranged to fit a small high speed drill (ca. 18,000 rpm). With the aid of a rubber stopper, these blades could be fitted into a small beaker which, in turn, was immersed in an ice bath. With this device, 30 to 60 seconds maceration gave an homogenate that showed mostly granular material of variable sizes, very few recognizable fiber fragments and some strands of connective tissue. Various other homogenizers were used during the first trials but all were unsatisfactory because of excessive contamination with Na or because of excessive frothing during grinding. Especially with all-glass, rubbing type homogenizers, contamination with Na was very pronounced. Even all-quartz apparatus could not be used for this reason.

Homogenates, following preparation, were allowed to equilibrate for 30 to 60 minutes in an ice bath (0° to 2°C.). They were then centrifuged in a refrigerated centrifuge for 10 minutes at 21,000g. Supernatants were then decanted and volumes of supernatants and residues determined. Residues were collected by resuspension in sucrose medium. Samples were taken of original homogenates, supernatants and residue suspensions for determination of Na, K and N. Nitrogen was determined by the usual micro-Kjeldahl distillation with collection of the ammonia in boric acid and titration with 0.01 HCl. Na and K were determined by the direct method with the Perkin-Elmer flame photometer, model 52-A, with frequent checks on standard solutions. Samples were prepared for Na and K analysis by pipetting known volumes into pyrex test tubes previously boiled with acid. One ml. of HNO<sub>3</sub> was then added per tube and the samples were digested at 100°C. for about one hour. The samples were then transferred to 15 ml. graduated centrifuge tubes and made up to volume. If visible sediments were present the tubes were centrifuged in an ordinary clinical centrifuge until clear<sup>2</sup>. Analyses were then carried out by feeding the diluted, digested samples into the flame photometer by means of a capillary tube siphoning arrangement. Reagent controls were carried through the whole procedure at frequent intervals and results were discarded if noticeable contamination was observed in the controls.

Contamination, especially with sodium, gave much difficulty in the earlier experiments. Use of acid-washed glassware, rubber gloves, and rigid control of

<sup>1</sup> Other suspending media were not used during the series of experiments to be reported here. The sucrose medium was chosen because of the success reported for the isolation of mitochondria with sucrose solution as a suspending medium (5). The strongly hypertonic solutions most effective for mitochondrial isolation were not tested.

<sup>2</sup> That no selective loss was incurred here is shown by the satisfactory recovery of both Na and K shown in table 2.

purity of reagents was essential. In order to make reasonably certain that contamination was not influencing the results, balance sheets were figured for each experiment, with the requirement that the sums of the ions of supernatant and residue should equal the calculated totals based on separate analyses of the original homogenates. This is recommended as a desirable criterion for any study dealing with the distribution of elements as ubiquitous as Na and K.

While the accuracy of the analyses with the flame photometer was excellent, it is obvious that the whole procedure for any given determination involves several steps of pipetting, dilution and digestion. All analyses reported here are the results of duplicate determinations, each being carried independently through the whole digestion and dilution procedure. Typically, for each system to be analyzed, one- and 2-ml. samples (3- and 5-ml. samples for residues) were pipetted to pyrex tubes and digested. After digestion, each was diluted to 15 ml. Thus determinations were made, not only on different amounts, but at different places on the flame photometer scale. This would tend to obviate purely mechanical errors. Averages, for inclusion in the tables, were then arrived at by adding amounts per 15 ml. and then dividing the sum by the combined volumes of samples taken. Partial protocols of two typical runs are given in table 1. Comparison of individual determinations with averages shows an average deviation of individual samples of around 5 per cent. If the averages are then compared (in these cases the 4 samples for each run represent quadruplicate runs of duplicate samples) it is seen that averages agree within at least 2 per cent. These determinations are on whole homogenates. The error was slightly larger for analyses of residues where the concentrations dealt with were about one-third of those figured in table 1.

Nitrogen analyses were carried out on most samples but will not be reported in detail. In round numbers, 70 per cent of the nitrogen was found in the residue fraction. This proportion did not vary significantly, even in the experiments in which added electrolytes were tested, and even when there was a wide variation in the volume of the residue. No added buffer was used. The pH values for whole homogenates and supernatants, as measured by the glass electrode, varied from 6.8 to 7.0 except in a few instances noted in the text.

#### RESULTS

Table 2 presents the results of 11 experiments. For ease of comparison, the results are given as percentages of amounts of Na or K of the original homogenate. For example, in experiment 2/23, the figure 64 under Na in supernatant means that the product, concentration  $\times$  volume of supernatant decanted, was 64 per cent of the product, concentration  $\times$  volume of original homogenate centrifuged. No corrections for contamination of residue by supernatant were made in obtaining these figures. In general, homogenates contained about 0.07 gm. muscle/ml. and the concentrations of ions calculated for whole muscle were, for Na, 24.1 and for K, 71.6 mEq/kg. wet weight.

Recoveries of Na and K, while showing some variation in individual experiments, are satisfactory and averages for the two elements are nearly identical. This agreement indicates that no systematic contamination during the centrifuging or subse-

quent treatment of the samples was influencing the results. Table 1 shows clearly that somewhat less than three-fourths of the original content of Na and K is contained in the supernatant of about four-fifths of the original volume. Thus there is, in effect, a dilution of the supernatant as compared to the original, the excess appearing in the residue. This would be expected if the residue contained base bind-

TABLE 1. PROTOCOLS OF 2 TYPICAL EXPERIMENTS

PREP. NO.	SAMPLE	SODIUM			POTASSIUM		
		conc. 15. ml. dil.	av. conc./ml. sample	diff. from av. ml. sample	conc. 15 ml. dil.	av. conc./ml. sample	diff. from av./ml. sample
	ml.						
1/31							
1a	1	110		1	345		1
1b	2	214	109	2	686	344	1
2a	1	114		4	340		0
2b	2	218	110	1	680	340	0
3a	1	106		4	345		13
3b	2	224	110	2	730	358	7
4a	1	105		1	350		5
4b	2	214	106	2	720	355	5
Mean			109	2		349	4
$\sigma'$			$\pm 1.9$			$\pm 8.6$	
$\sigma'_m$			$\pm 0.9$			$\pm 4.3$	
2/16							
1a	1	112		2	310		3
1b	2	214	110	3	610	307	2
2a	1	104		7	310		25
2b	2	230	111	4	684	335	7
3a	1	130		15	345		5
3b	2	214	115	8	704	350	2
4a	1	121		8	330		18
4b	2	218	113	4	716	348	10
Mean			112	7		337	9
$\sigma'$			$\pm 2.2$			$\pm 20$	
$\sigma'_m$			$\pm 1.1$			$\pm 10$	

Whole muscle homogenates were divided into 4 portions each, and then each portion analyzed by running one and 2 ml. samples through the entire digestion, dilution and flame photometer procedure. Concentrations are  $\mu\text{M/l.}$  Muscle concentrations of the original homogenate: for 1/31, 0.077 gm/ml.; for 2/16, 0.083 gm/ml.

ing groups. On the other hand, the results also show that there is no marked selective retention by the residue. Amounts collected in the two fractions, supernatant and residue, for both Na and K, agree within a few per cent.

Further examination of the data shows, however, that there are small consistent differences to be seen. On the average, the residue contains a slight excess of sodium and the supernatant a slight excess of potassium. The differences, as listed in table 2, are not statistically significant but the consistency of variation is suggestive.



At least one source of variation, which would decrease the statistical reliability of the results, resides in the variable volumes occupied by supernatant and residue. If the assumption is made that the solid material of the residue occupies negligible volume (ca. 20% of 0.07 ml/ml.) then the amounts of elements in the entire supernatant (that decanted plus that remaining as contaminant of residue) can be calculated by multiplying the amount of element in the decanted supernatant by the ratio total volume/decanted volume. If this is done, for the data shown in table 2,  $91 \pm 1.2$  per cent of the Na is found in the supernatant,  $94 \pm 1.0$  per cent of the K. The difference between the two has a *P* value of less than 0.01 as calculated by Students method (6) for comparison of means of small samples. Thus there is sig-

TABLE 2. ANALYSIS OF SUPERNATANT AND RESIDUES OF MUSCLE HOMOGENATES CENTRIFUGED 10 MINUTES AT 21,000 G

EXPER. DATE	Na			K			VOLUME OF RESIDUE
	SUPERNATANT	RESIDUE	RECOVERY	SUPERNATANT	RESIDUE	RECOVERY	
							%
2/23	64	35	99	68	34	102	29
2/22	81	23	104	80	20	100	16
2/22	76	25	101	74	21	95	16
2/21	60	31	91	68	33	101	27
2/16	67	29	96	69	27	96	24
1/28	82	26	108	78	25	103	20
1/31	67	36	103	73	36	109	26
12/31	74	24	98	80	23	103	15
12/27	69	34	103	70	32	102	23
12/12	70	37	107	74	33	107	23
12/13	80	25	105	81	17	98	12
Av.....	72	29	101	74	27	101	21

Figures represent fractions (as %) of Na and K recovered. For example, 15 ml. of whole homogenate of experiment 2/23 had an Na concentration of 1.5 mm/l. or contained 22.5  $\mu$ M of Na. The volume of the supernatant was 10.7 ml. with an Na concentration of 1.35 or 14.4  $\mu$ M of Na. The supernatant thus contained 64 per cent of the total Na.

nificantly less Na in the supernatant. The reverse situation can be shown to hold for the residue.

If it is assumed that supernatant and residue represent the two phases of a system in diffusion equilibrium, then Donnan ratios may be calculated. Using the concentrations in the two phases for the experiments shown in table 2, the Donnan ratio (conc. residue/conc. supernatant) for Na is  $1.54 \pm .059$  and that for K is  $1.37 \pm .031$ . The difference between the two, 0.17, has a *P* value of about 0.01 and is thus significant.

Analysis of the results from several different points of view thus shows a significant excess of Na in the residue. While it is theoretically possible that this could be due to a K binding in the supernatant, this explanation seems highly unlikely since 70 per cent of the solid material of the tissue is in the residue and binding might be most expected associated with this material. A sodium binding of about

3 per cent of the total in the tissue or slightly less than one mm/kg. of muscle would account for the results. The further experiments reported here on ion displacement from the residue material are based on the assumption that the small fraction of excess sodium is significant.

The insoluble residue presumably contains base binding groups of proteins and a variety of complexes of proteins with other materials. These groups, when sedimented, would carry with them sodium and potassium, presumably in an equivalent ratio determined by the respective equivalent fractions and by any specific binding capacity for an individual ion species. Addition of extra cation, thus altering the equivalent fractions of Na and K, should displace these elements from the sedimentable fraction and any specific binding should appear in the character of the ion ratios of retained and displaced ions. Accordingly, experiments were carried out in which  $\text{NH}_4\text{NO}_3$ ,  $\text{CaCl}_2$  and  $\text{MgCl}_2$  were added to the freshly prepared homogenates. After incubation, under standard conditions, the homogenates were centrifuged and analyzed as usual. Since interest centers in the binding of ions by the insoluble residue, these results are calculated as excess ion concentrations in the residue and as excess ion concentrations per unit of excess nitrogen. Again, the simplifying assumption is made that all but a negligible portion of the volume of the residue is contamination by supernatant. Hence concentration excesses were calculated simply by subtracting the concentration of Na, K or N in the supernatant from the concentration in the residue. Concentration excess of Na or K, divided by concentration excess of N, gives excess element per unit of nitrogen.

In calculating theoretical ion excess concentrations in the presence of known amounts of added electrolyte it was assumed that the sum,  $[\text{Na}] + [\text{K}]$ , was a fair measure of total normal cation content. Assuming that Na and K combined with the nitrogen excess according to their equivalent fractions, then the expected concentration excess of Na or K in the presence of extra ion would be found by multiplying the control concentration by the ratio  $[\text{Na}] + [\text{K}]/[\text{Na}] + [\text{K}] + [\text{X}]$  where  $[\text{Na}]$  and  $[\text{K}]$  are found by analysis and  $[\text{X}]$  is the equivalent concentration of added cation<sup>3</sup>.

Table 3 gives results of experiments on the displacement of Na and K from the residue by added cation. Considering displacement by  $\text{NH}_4$  first, it is apparent that the concentration excess found in the presence of  $\text{NH}_4$  is of the correct order of magnitude but slightly higher than that calculated for both Na and K (table 4). The original Na/K ratio was 0.4, the ratio for retained ions is 0.5 and for the displaced ions is 0.3 (table 4). Thus relatively more K is displaced than Na, confirming the assumption that Na is selectively bound by the residue.

In the presence of excess calcium, the selective retention of sodium is accentuated while with magnesium selectivity is apparently decreased. The statistical significance of the differences between effects of ions is not high however, although the deviations from normal distributions are.

An incomplete experiment involving the addition of excess acid ( $\text{HNO}_3$ , 0.08

<sup>3</sup> If this were a true exchange, similar to that observed in soils (8), the simple equivalent fraction used would hold only for monovalent ions in the absence of selective binding. More detailed analyses, however, would involve additional assumptions about the nature of the material being dealt with.

in final concentration) shows (table 3) drastic displacement of both Na and K with an average ratio for retained elements of Na/K = 0.38.

TABLE 3. FIGURES FOR CONCENTRATION EXCESS (SEE TEXT) OF Na, K AND N, FOR RESIDUES OF CENTRIFUGED MUSCLE HOMOGENATES AND CONCENTRATION EXCESS PER UNIT OF EXCESS NITROGEN FOR Na AND K

EXPER. DATE	Na	CONC. EXCESS K	N	CONC. EXCESS/U Na	EXCESS NITROGEN K	VOLUME RESI- DUE TOTAL
						%
Normal						
2/23	6.5	16.2	41	16	40	26
2/21	5.1	10.5	33	15	32	28
2/16	5.9	12.7	46	13	28	23
2/2	6.5	15.8	36	18	44	16
1/31	7.0	19.0	40	17	47	27
1/28	5.0	12.0	42	12	29	20
Av.....	6.0	14.4	40	15	37	23
CaCl <sub>2</sub>						
2/16	6.0	14.0	131	5	11	8
2/2	9.2	13.2	105	9	13	11
1/31	9.0	13.0	99	9	13	13
Av.....	8.1	13.4	112	8	12	11
MgCl <sub>2</sub>						
2/21	3.0	12.0	100	3	12	9
2/2	7.4	10.5	98	8	11	10
1/31	7.0	13.0	153	5	9	9
Av.....	5.8	11.8	117	5	11	9
N <sub>2</sub> NO <sub>3</sub>						
1/31	5.0	13.0	72	7	18	18
1/28	8.0	18.0	78	10	23	10
1/22	6.5	10.0	55	12	18	19
Av.....	6.5	13.7	68	10	20	16
HNO <sub>3</sub>						
2/22	2.5	4.0				7
2/22	2.2	8.0				7
Av.....	2.3	6.0				7

Concentration excess for Na and K, for N, mg/l. Concentration excess for each u nitrogen: mm/gm. nitrogen. Salts added where indicated to a final concentration of 0.01 N; HNO<sub>3</sub>, final concentration 0.08 N.

Figure 1 shows the relationship between concentration excess per unit of nitrogen and various concentrations of extra added cations. The dotted lines indicate the theoretical concentrations expected if the added ions displaced Na and K according to their equivalent ratios. It is readily apparent from this chart that the displacement by NH<sub>4</sub>, while less than expected, follows essentially the predicted course, assuming a small portion of bound sodium. Calcium and magnesium, on the other hand, are

considerably more effective, especially at the lower concentrations, magnesium tending to abolish selective retention of sodium.

Added electrolytes all cause a volume diminution of the residue although there is no significant change in the total nitrogen of the residue. Here again, the alkaline-earth metals are more effective than is  $\text{NH}_4$ . There is a suggestion in the data of a significant parallelism between amount of Na and K removed and the degree of volume constriction of the residue. Presumably the volume changes relate to the inclusion of the acto-myosin complex in the residue material. Whether the decrease in ionic content of the residue relates to a change in base binding groups of the contracting material, or a mechanical squeezing out of some substance cannot be determined from the present data.

A few experiments were made in which homogenates or supernatants were dialysed in a rocking dialyser in the cold. Cellophane tubes, about  $\frac{3}{4}$  inches in diameter and about 8 inches long, were mounted in a rocking glass cylinder and so arranged that samples could be withdrawn from inside and outside the dialysis sac at intervals.

TABLE 4. SUMMARY OF AVERAGE RELATIVE CONCENTRATION EXCESS OF Na AND K/U NITROGEN, DISPLACED AND RETAINED IN PRESENCE OF ADDED  $\text{Ca}^{++}$ ,  $\text{NH}_4^+$ , AND  $\text{Mg}^{++}$

	NORMAL	$\text{NH}_4\text{NO}_3$ DIS- PLACED-RETAINED		$\text{CaCl}_2$ DISPLACED- RETAINED		$\text{MgCl}_2$ DISPLACED- RETAINED		CALCULATED DIS- PLACED-RETAINED	
Na	100	33	67	47	53	67	33	60	40
K	100	46	54	68	32	70	30	60	40
Na/K	0.40	.30	.50	.28	.67	.38	.45	.40	.40

Figures from data of table 3 and, for Na and K, represent percentage displacement or retention as compared to the normal controls. Na/K ratios calculated from the figures of table 3.

Routinely, 30 ml. of material was placed inside the sac and 60 ml. of suspension medium (0.2 M sucrose) outside at the start. The cellophane was washed for several days with many changes of distilled water before using. Cellophane, washed a few hours until apparently sodium free, would still release considerable amounts of Na when treated with dilute KCl solution, at the same time binding potassium.

Immediately upon placing the solutions in the dialysis apparatus, the whole was shaken by hand for about one-half minute and then the zero time samples taken. This was found necessary in order to allow the solutions to come to equilibrium with the ion exchange system of the membrane. In the example given in table 5, 48.5  $\mu\text{Eq.}$  of Na and 143  $\mu\text{Eq.}$  of K were added in the 30 ml. of homogenate. After the preliminary equilibration, analysis of both inside and outside solutions showed that about 21  $\mu\text{Eq.}$  of K had disappeared from the fluid phases and about 6  $\mu\text{Eq.}$  of Na. These amounts did not reappear during the 4-hour period of dialysis but, in other experiments, could be shown to be bound to the cellophane, easily released by treatment with, for example, dilute acid.

The data of table 5 do not show any significant binding of either ion. However, this is consistent with the findings on the fractions of centrifuged homogenates which show only a small portion of the total Na bound to the insoluble fraction, an amount which could be masked in the dialysis experiments by a small uncom-

pensated selective binding by the cellophane system itself. On the other hand, if potassium (present in larger amounts) had been bound to some colloidal constituent of the soluble material, such a condition might have been expected to show up in these experiments. Dialysis of supernatants also showed no significant selective retention of either Na or K. Further dialysis (or ultra-filtration) experiments were not attempted because of the methodological difficulties occasioned by the cellophane ion exchange system. Electrodialysis might be of value in investigating the possible existence of non-ionized, diffusible, complexes of Na or K.

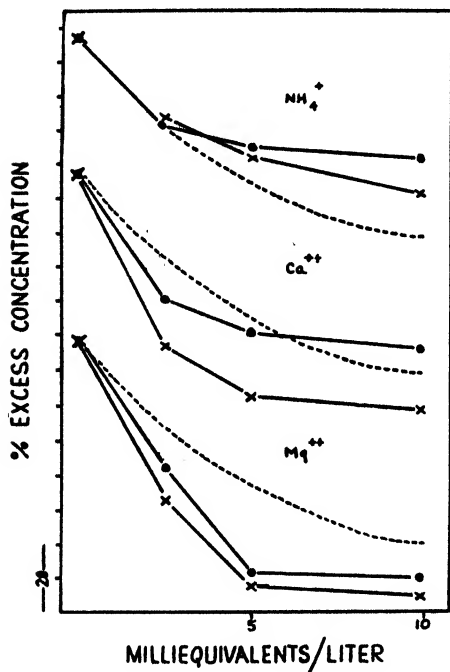


Fig. 1. RELATIVE EXCESS concentration/unit nitrogen plotted against equivalent concentrations of  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , and  $\text{NH}_4\text{NO}_3$  added to homogenates. Normal values were taken as 100 per cent (table 3) and curves displaced along vertical axis to avoid overlapping. Dotted lines represent calculated values for concentration excesses, assuming only univalent cations with no selective binding. Normal points, averages of 6 to 10 determinations, points at 10 mEq/l., averages of three determinations each (table 3). Other points, single determinations. Dots represent Na, crosses represent K.

#### DISCUSSION

As Michaelis has pointed out (7) 'binding' of such elements as sodium and potassium in an aqueous medium is difficult to visualize. Nevertheless, the fact remains that cells preferentially hold potassium or remove sodium. Either process implies a chemical selectivity, the exact nature of which remains to be discovered. However, a matter of first importance is to examine whether or not a preferential binding, no matter how unknown in nature, does exist that can be demonstrated in components of the cell as well as in the intact living system.

In order to be of any value in explaining potassium accumulation, potassium binding would necessarily be of a reasonably high magnitude. Appreciable selection of K by muscle brei components certainly is not evident in the experiments described here. On the other hand, sodium binding in cells, to serve as an exchange and/or

transport system, might be expected to occur in quantitatively small amounts, as has been demonstrated for the breis<sup>4</sup>. It remains for future work to determine whether or not the sodium binding does occur at the surface, where it can be of use in accounting, especially, for the high turnover rate of Na, and to gain information about its nature. Suggestive phenomena which may be pertinent to the ion balance of living cells are to be found in base exchange systems of soils (8).

In view of the apparent low rate of penetration of ionic sodium (3) as contrasted with the high rate of exchange (1) it would seem probable that any sodium combination at the surface would be essentially non-ionic. The high electrical resistances encountered at normal cell surfaces would also indicate this. If this is true, then such a non-ionic sodium exchange system could become a transport system if either the formation or the destruction of the complex were coupled to energy-yielding reactions. Again, in view of the exchanges noted, formation of low conductance sodium areas of the cell surface might seem indicated, maintained by

TABLE 5. CONCENTRATIONS (mm/l.) INSIDE AND OUTSIDE DIALYSIS SAC OF CELLOPHANE

TIME	Na			K		
	INSIDE	OUTSIDE	IN OUT	INSIDE	OUTSIDE	IN OUT
min.						
0	1.40	0.03		4.05		
15	1.17	0.09	13	3.22	0.225	14
30	0.93	0.15	6.2	2.73	0.45	6.1
90	0.79	0.27	2.9	2.28	0.73	3.1
180	0.66	0.33	2.0	1.90	0.90	2.1
240	0.60	0.39	1.5	1.65	0.97	1.7

Rocking dialyser, 30 ml. muscle homogenate inside sac, 60 ml. 0.2 M sucrose outside at zero time. One- and two-ml. samples removed from inside and outside, respectively, at times indicated.

the energetic formation of the special complex. Destruction of the energy linkage (e.g. by stimulation?) would thus have the effect of suddenly increasing the ionic permeability, since the complex is no longer formed, with non-selective leakage of sodium inward and potassium outward. It might also be suspected that the many esterases demonstrated at the outer boundaries of some cell systems (9) might play a role in the return of  $\text{Na}^+$  to the outside during transport.

One of the few suggestions that could be made about the general nature of sodium binding would derive from studies showing that metaphosphates bind Na (10). A polyphosphate, analysis corresponding to the empirical formula  $\text{KNa}_2(\text{PO}_3)_3$ , dialysed against HCl loses all K but no Na. Whether this property is shared by other

<sup>4</sup> The order of magnitude of sodium binding reported in this paper would be consistent with surface localization. Assuming 80 per cent fiber volume of the muscle and an average fiber diameter of about  $100\mu$ , the total surface/gm. is of the order of  $300\text{ cm}^2$ . If each gm. of muscle contains one  $\mu$  M of bound Na, then there is on the order of  $50\text{ Å}^2$  surface for each Na atom. Thus, there is plenty of space available. Ussing (1949), estimates a total flux of Na as about  $0.03\text{ mm/kg/hour}$  or on the order of  $1.10^{11}$  atoms Na/second. Since over  $10^{16}$  atoms of Na are bound, no very high turnover rate per atom need be postulated.

polyphosphates is not known. On the basis of behavior of whole cells, many phosphorylated intermediates have been implicated in the Na—K balance of cells (11). In the absence of direct studies, the correlation of any such compounds with K accumulation could mean either association with K retention or Na removal. Some time ago, the parallelism between K content and creatine of muscles was pointed out (12). A substance such as creatine might serve either to furnish many negative charges to allow  $K^+$  to exist, or as part of an energetic Na removal system.

At present, it is difficult to state, with precision, at what rate energy must be expended to cause a net transport of Na outwards to compensate for the inward leakage due to the high gradient existing between muscle fiber and environment (1). Hence a precise statement of the work that must be accomplished by a selective sodium system cannot be made. The studies reported here show a small selective retention of Na by the sedimentable residue. This could be due to binding by the insoluble residue, or to a partial binding of a soluble non-ionic sodium form (4). The fractionation procedures used do not distinguish between soluble ionic and non-ionic sodium or potassium if they exist. Electrophoretic separations would be of value in investigating this point.

#### SUMMARY

The sedimentable fraction of a frog muscle homogenate is shown to contain a slight excess of sodium as compared to potassium. Both Na and K associated with the sedimentable material are displaced by  $NH_4NO_3$  added to the whole homogenate, relatively more K being displaced than Na.  $CaCl_2$  and especially  $MgCl_2$  are more effective than  $NH_4NO_3$  in displacing K and Na. Displacement by  $MgCl_2$  appears equally marked for both Na and K.

No evidence is found by centrifuged separation of components of a frog muscle homogenate for potassium binding. Dialysis experiments likewise give no evidence for K binding, providing that the ion binding of the dialyzing membrane is taken into account. The results are interpreted as evidence for the binding of a small (2–3% of the total) amount of sodium. Possibilities that this bound sodium serves as an exchange and transport system at the muscle fiber surface are discussed.

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# EFFECT OF IODOACETATE ON CHANGES IN MUSCULAR LATENCY INDUCED BY ACTIVITY<sup>1</sup>

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THE mechanical latent period (LP) of muscular contraction, as studied in this paper, is the time interval between the instant of application of a direct stimulus to the muscle fibers and the instant at which the resultant tension onset is just observed. When the piezoelectric, cathode-ray oscillographic technique (1) is used to record isometric tension changes, it is easily demonstrated that the LP consists of an initial mechanically quiescent phase which is followed by a minute precontractile relaxation, the latency relaxation (LR).

In an earlier publication (2) dealing with the normal frog sartorius, it has been shown that activity causes marked alterations in the events of the LP. A 3-second tetanus, e.g. causes immediate reductions in both the duration and the depth of the LR which are gradually and finally completely reversed during post-tetanic recovery of about 45 minutes. The activity has no influence on the duration of the first, i.e. the mechanically inactive part of the LP. Other kinds of action, such as a series of twitches, or prolonged contraction to the point of fatigue, result in different modifications of the LP which will be discussed later.

Full analysis of the results obtained with various types of activity demonstrated that the temporal changes of the LP run closely parallel to the pH modifications developed in the muscle by its own activity, i.e. the higher the pH, the shorter the LR phase of the latent period. Thus some process localized in time during the LR occurs more rapidly, the more alkaline the muscle. Since this process takes place just prior to observable contraction, it was inferred that tension development depends on some reaction which is speeded up by increase in pH. It was hypothesized that this reaction is the hydrolysis of adenosinetriphosphate (ATP), since, among the known chemical changes most probably associated with the direct energization of the contractile material, the breakdown of ATP is unique in having a pH dependence parallel to that which holds for tension onset.

The pH changes in activated muscle (e.g. 3-5) are principally dependent on glycolysis and the decomposition of creatine phosphate (CP). After moderate activity, muscles become relatively alkaline partly because lactic acid formation is slight, or absent, and especially because the creatine released is a quite strong base;

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and this change is reversed as recovery goes on. The present studies of muscles treated with iodoacetate (IAA) are of interest because this substance inhibits lactic acid formation and in consequence of this causes CP to split irreversibly. Thus the accompanying pH rise is irreversible. If the previously determined correlation between pH and speed of tension onset holds for the poisoned muscle, activity should result in a normal, but irreversible reduction in the duration of the LR phase of the latent period. Within certain limits the current results are in conformance with this prediction.

Another chemical feature of IAA muscles is that, after sufficient activity, at the time when the store of CP has been depleted, ATP breaks down irreversibly. The latency behavior of muscles in this state is of interest in relation to the view that the development of tension is dependent on the hydrolysis of ATP. Although the present results are not perfectly clear in regard to this point, they are consistent with the hypothesis that ATP plays some mechanochemical role during the latent period.

#### METHOD

All experiments have been made on directly stimulated sartorius muscles of medium- to large-size *Rana pipiens* at temperatures in the range from 21.3° to 24.0° C. Although the muscles were in general not curarized, direct stimulation was always obtained by using a sufficiently high shock strength and noting that the LP duration as seen on the cathode-ray screen did not include the time for transmission of excitation (of the order of 2 msec.) across neuromuscular junctions. For further details of this point see previous papers (1, 2). In any case, several experiments on curarized IAA muscles gave similar results to those for the uncurarized but directly stimulated IAA preparations. Except for certain variations to be noted later regarding the IAA muscle, the general procedures for determining the latency alterations caused by activity were exactly like those previously described (2).

In all the experiments of the present studies the stimulating electrodes were placed at the ends of the muscle, with the excitatory cathode in contact with the upper (tibial) end at the point of connection of the muscle to the fine metal chain that leads to the pickup-stylus. In view of the position of the cathode, the initial mechanical response at the tibial end of the muscle was transmitted directly to the chain leading to the stylus, and it cannot represent any effect of a propagated mechanical wave that might arise if the cathode were placed at a spot on the muscle some distance away from the point of connection of the muscle to the metal chain. Since the first change that appears in our records corresponds to a relaxation of the muscle, it must be concluded that this alteration—the latency relaxation—is the first mechanical response to stimulation. Thus, these considerations preclude the view that the LR is an artifact resulting from a preceding contractile change, as claimed by Schoepfle and Gilson (6), since this interpretation of the LR critically depends upon an assumed peculiarity in muscle of the transmission of a compressional wave from its point of origin at the cathode to a distant point at which registration is effected—a type of transmission that is absent from the system that produces our LR records. Still other evidence contrary to the Schoepfle-Gilson artifact interpretation of the LR will be found in other publications (7-9).

## RESULTS

Figure 1 presents a typical record of our experiments and indicates the symbols used to designate the relevant variables. The cathode-ray trace, upon which is superimposed a 10,000 cycles/second timing wave, corresponds to the isometric tension changes of the first few milliseconds of the maximum twitch mechanical response. The beginning of the sweep at the left coincides with the instant of stimulation, the stimulus being a thyatron controlled condensor discharge with a time constant of about 0.1 msec.  $L_R$  measures the duration of the mechanically inactive portion of the LP; it will be referred to as the relaxation latency.  $L_0$ ,  $L$ , and  $L_1$  represent suc-

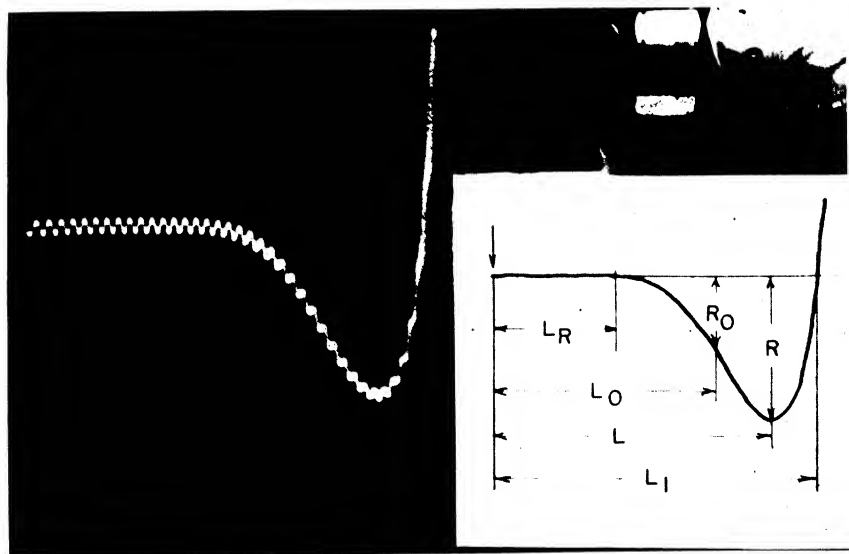


Fig. 1. REPRODUCTION OF TYPICAL LATENT PERIOD and tension response of maximal isometric twitch of frog sartorius muscle as registered on a cathode-ray oscillograph. (Retouched.) *Main curve*, having superimposed timing wave of 10,000 cycles/second, represents latency mechanical changes. *Inset* defines symbols of latency parameters. *Upper and lower bands of light* in top right corner give, respectively, initial and total tension of the twitch. See text for further details.

cessive intervals of time associated with the onset of tension. Obviously,  $L$  measures the mechanical latent period, i.e. the tension latency, as we have already defined this variable. But, in the current experiments, as well as in earlier work (1, 2),  $L_0$  and  $L_1$  always vary in parallel with  $L$ . It is especially noteworthy that  $L_0$  behaves in this way, for this means that the mechanism directly connected with tension onset is already in operation at this moment even though the actual muscle behavior at this time is relaxation. However, for methodological reasons, we will use  $L$  to represent the LP, partly because phenomologically it indicates the first instant of directly observable tension output, and partly because it is most easily and precisely measurable.  $R$  signifies the depth of the LR. Finally, the bands of light in the upper right corner of the figure give a record, obtained by a conventional optical lever method, of the initial and total developed tensions. The upper beam position

gives the initial tension and the downward deflection measures the developed tension output, which will be symbolized by  $T$ .

*Effect of a Single Tetanus.* We are concerned here with the alterations induced by a directly stimulated, maximal, 3-second isometric tetanus at a frequency of 45 shocks/second. The average behavior of six IAA muscles will be compared with that of six normal muscles. After excision, all the muscles were soaked for one hour in oxygenated Ringer's solution buffered with phosphates to pH 7.1. In the case of the treated muscles, 1-80,000 IAA (Eastman) was added to the Ringer's bath. With the completion of the soaking period, each muscle was set up in a moist chamber and connected to the stylus of the pickup at an initial tension of 3 gm. After equilibration to the constant temperature of the water bath into which the moist chamber was placed, two responses to maximal twitch shocks, separated by four minutes, were recorded to obtain the rested behavior of the muscle. Next, the tetanus shock was applied, and after this, maximal twitch responses were recorded, usually at the following times, measured from the termination of the tetanus: 5 and 30 seconds, and 1, 2, 3, 4, 5, 7, 10, 15, 20, and 30 minutes. Some post-tetanic runs did not exactly adhere to this schedule, and in such cases interpolated values at the above instants were obtained from graphs of the actual results. No error of any consequence was introduced by this procedure.

The initial tension, with an exception noted below, was kept constant at 3 gm. for all the IAA as well as the normal responses, by manual adjustment of a device for regulation of the initial tension of the muscle. Only irregular minor adjustments were necessary during the normal runs, but the IAA muscles developed typical pronounced rigor sometime after tetanus, and since the initial tension can itself modify the latency response (1), as well as other muscular behavior, the progressively developing rigor was nullified just before each post-tetanic twitch by restoring the initial tension to the standard 3-gm. value. However, due to time limitations, this could not be done for the test twitches at 5 and 30 seconds, and at 1 minute following the tetanus. Excepting this set of early responses, a measure of the IAA rigor developed up to any moment was obtained by recording the individual diminutions in initial tension required to maintain the constant 3-gm. value, and summing these reductions introduced up to that instant.

Figure 2 presents the results for the tension variables of the two sets of muscles. The horizontal axis measures the time following tetanus, with the zero point indicating the instant of termination of the tetanus. Each point plotted for a given variable is the average for the percentage values of that variable in the six relevant muscles, at the indicated post-tetanic moment, relative to the average for that quantity just preceding the tetanus. No pre-tetanic values are plotted, but, for all the variables, these would be 100 per cent. Exceptions to the above procedure obtain for the IAA rigor points, these being compared to the average pre-tetanic developed twitch tension of the IAA muscles.

Our main concern at this moment is with the latency tension change represented by  $R$ . But it is of interest, first, to study the developed twitch tension,  $T$ , and the development of the IAA rigor as a means of following by more usual criteria the post-tetanic state of the muscles. The normal muscle shows an immediate poten-

tiation of its twitch tension output to about 135 per cent of the pre-tetanus value; this rises slightly, but significantly, for 30 seconds, and then it falls progressively until at 30 minutes after tetanus, T is quite normal again. The IAA muscle exhibits a somewhat similar course for T, but the potentiation is less than in the normal muscle and it disappears more rapidly, so that after some 3 minutes, the twitch output is progressively decreasing to values below that of the rested IAA muscles. The initial tension of the normal muscles (not plotted) is essentially constant, but that of the IAA muscles shows the characteristic iodoacetate rigor rise following activity. (It is noteworthy, however, that the initial tension of the IAA muscle falls

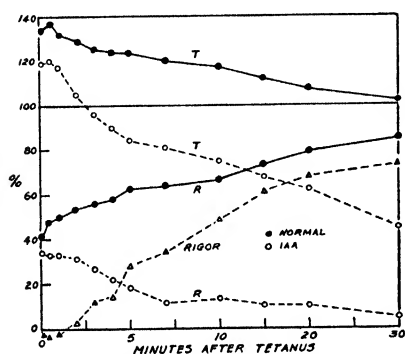


Fig. 2 (left). AVERAGE EFFECTS of 3-second maximal isometric tetanus on behavior of tension variables in subsequent twitch responses of normal and IAA (1-80,000) muscles. T, developed tension; R, depth of latency relaxation. *Abscissae*, times following termination of tetanus. *Ordinates*, relative outputs in per cent, with 100 per cent representing pre-tetanic value for each parameter, excepting IAA rigor which throughout is compared to developed tension of pre-tetanic twitch. Average absolute values corresponding to 100 per cent pre-tetanic behavior: T (quite variable depending on size of muscle), about 41 gm.; R (also quite variable), about 0.05 gm. Average of lumped standard errors of normal and IAA post-tetanic points is for T,  $\pm 2.2$  per cent, and for R,  $\pm 5.0$  per cent.

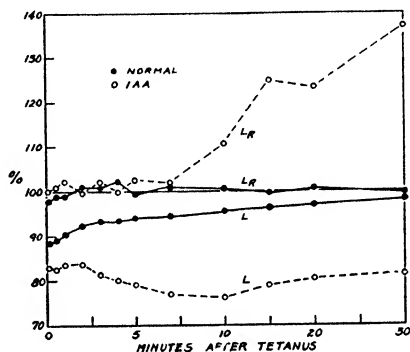


Fig. 3 (right). AVERAGE EFFECTS of 3-second maximal isometric tetanus on latency time parameters in post-tetanic twitches of normal and IAA muscles.  $L_R$ , latency of latency relaxation; L, latency for tension output. (See fig. 1 and text for details of significance of symbols.) *Abscissae and ordinates* as in fig. 2. Average absolute values for both normal and IAA muscles before tetanus:  $L_R = 1.12$  msec.,  $L = 2.70$  msec. Average of lumped standard errors of normal and IAA points is for  $L_R$ ,  $\pm 2.6$  per cent, and for L,  $\pm 1.3$  per cent.

immediately after the tetanus by about 1.5 gm., an observation that merits further study, since previous notations of the resting tension in activated IAA muscles have always emphasized only the subsequent rigor development.) Thus, as indicated by the behavior of T and the rigor development, the tetanized IAA muscles, in contrast to the normal, are becoming increasingly moribund, and at about 45 to 60 minutes after the tetanus they fail to respond in any way when stimulated.

We now note that R, following tetanus, undergoes a reversible decrease in normal muscle, but it is irreversibly reduced in IAA muscle. In the poisoned condition, the initial decrease in R (65%) is somewhat greater than in the normal (58%), and after remaining virtually constant during the first minute or two after tetanus, when the T is still potentiated and the initial tension is reduced, R then diminishes further

as the muscles depart more and more from their healthy state. Some individual muscles exhibit a slight increase in  $R$  during the first minute after tetanus which is followed by a later reduction as presented in the figure. This behavior, however, is masked in the figure by the average values plotted therein.

Figure 3 presents a comparison of the course of the latency time variables after tetanus.  $L_R$  in both poisoned and normal muscle is essentially constant during the first 5 to 7 minutes after the activity, but thereafter, while the normal  $L_R$  maintains its constancy, the IAA  $L_R$  rises steeply. The tension latency,  $L$ , shows a greater immediate decrease in the IAA muscle (16%) than in the normal (12%), and this reduction, paralleling the behavior of  $R$  in the same muscles, does not change appreciably during the first two minutes after tetanus. After this interval,  $L$  continues to decrease up to about the 10-minute point and then after this it rises slightly.

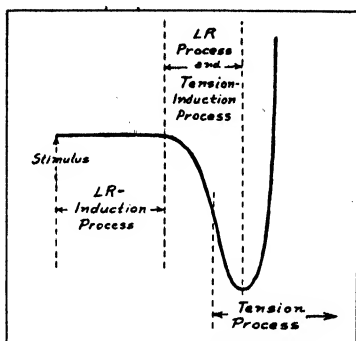


Fig. 4 (left). DIAGRAM of latency mechanical changes showing temporal correlation of various processes inferred to occur during latent period.

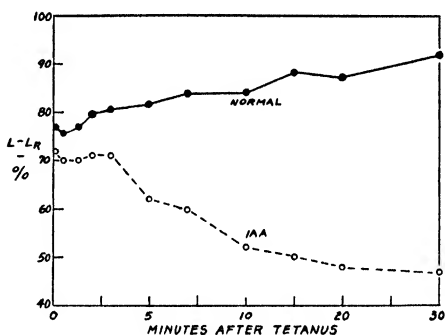


Fig. 5 (right). AVERAGE CHANGES of interval  $L-L_R$  following 3-second tetanus. Abscissae and ordinates as in figs. 2 and 3.

*Other Types of Activity.* In the paper (2) concerned with the responses of normal muscles, results were presented of experiments involving a 3-minute series of twitches at the rate of 20/minute and associated recovery, and a long series of 2-second tetani at 7-second intervals continued until the muscle was thoroughly fatigued. Similar studies have been made of muscles poisoned with IAA, with results that add nothing critically different from those already presented here. For example, in consequence of the long series of tetani,  $R$  progressively decreases, but it does so more quickly in the IAA muscle. Also in this type of experiment, the value of  $L$ , for both normal and IAA muscles, progressively decreases in response to the first four or five tetani and thereafter increases. More work needs to be done to determine whether there is any consistent difference between normal and IAA muscles in respect to this behavior of  $L$ ; but the results obtained to date may be interpreted within the general mechanism of the effects of IAA that will be presented later in the discussion section of this paper.

Some features of the 3-minute twitch activity series may be noted. In the normal muscle the first 3 or 4 twitches cause a slight progressive rise in  $L$  and, as further

activity occurs,  $L$  then decreases until at the end of the twitch series it is less than it was before the activity. This diminution of  $L$  continues somewhat into the first few minutes of the post-activity period; but after a recovery period of about 30 to 45 minutes the original rested value of  $L$  is restored. Throughout this type of experiment  $L_R$  remains essentially constant. As for  $R$ , it decreases progressively during the activity period and then immediately thereafter begins the process of reversal of this fall characteristic, in general, of the normal recovery period. In the identically investigated IAA muscle somewhat similar behavior is found. But, at the termination of the activity period, the decreases in  $L$  and  $R$  are larger than in the normal case, and they are more slowly and less completely reversible by rest, and the characteristic continued decrease of  $L$  into the early part of the recovery period is larger and more prolonged. These differences between the IAA and normal behavior are of the same general type as those obtained in response to a 3-second tetanus. But they are not so pronounced, as might be expected since the activity of a 3-minute series of twitches at 3-second intervals is much less than that of a 3-second tetanus with shocks at the rate of 45/second.

One feature of the 3-minute twitch series that has special interest is the essential identity of the initial reversible rise in  $L$  in both the IAA and the normal muscles. This shows that IAA does not affect the latency behavior until the muscle has undergone a relatively large amount of activity. In fact, comparisons of the latency events in the pre-activity responses of our normal and IAA muscles demonstrate that they are not significantly different. Henriques and Lundsgaard (10) had already noted this concerning the duration of the latent period, although their technique involved only simple kymographic recording of indirectly stimulated twitches. Thus, IAA does not exert its effects on the latent period directly, but does so only in combination with the consequences of at least a certain minimum amount of muscular activity.

#### DISCUSSION

Our results prove that the effects of activity on the latent period parameters  $L$  and  $R$  in the iodoacetate-poisoned muscle are more pronounced and, in general, irreversible, when compared to those found in normal tissue. Although this is true in more or less degree for all kinds of activity studied, it is most clearly demonstrated in experiments involving an intense burst of activity such as the 3-second tetani used to obtain the bulk of our data. It is noteworthy, however, that the relaxation latency ( $L_R$ ) is not immediately altered by activity in either the normal or the poisoned muscles. In the normal recovery period it continues to show no change, but in the IAA case it increases during the last 22 minutes of this period. An especially noteworthy observation of these experiments is the maintenance with only minor variations of the reduction in  $L$  of the IAA muscles throughout the post-tetanic period even though the concomitant progressive reduction in the twitch tension output ( $T$ ) and increase in rigor show that the muscle is approaching a completely moribund condition. Thus, despite the badly failing condition of such muscles, they nevertheless are able to respond with a shorter latent period, i.e. initiate their tension development more rapidly than is characteristic of similarly treated normal muscles.

In previous papers (1, 2) evidence has been presented demonstrating that the latent period as measured by  $L$  comprises three mechanically distinguishable events as illustrated in figure 4. The first is the *LR induction process* during which the muscle does not in any way change its tension, and whose duration is therefore given by  $L_R$ . This passes into the *LR process*, indicated by the characteristic slight latency reduction in tension, which then transforms into the *tension process* that is finally manifested in the usual increase of tension of the contraction period. Evidence was also adduced to indicate that the LR process may be identical with a *tension induction process*, and furthermore, that the tension process is already in operation during the LR (or tension induction) process even though our records show that the muscle is relaxing at this time.

Our present results give some indication of the effects of IAA in tetanically activated muscles on the behavior of these processes in subsequently excited twitch responses. In the IAA muscle subjected to a 3-second tetanus there is no alteration in  $L_R$  in the first 7 minutes of the post tetanic period and this behavior duplicates that of the normal muscle. But following this early interval  $L_R$  rises more or less in parallel with the increase in rigor and the large decrease in twitch-tension, which changes do not take place in the normal tissue. The progressively increasing magnitude of  $L_R$  during this later interval evidently reflects a slowing of the LR induction process. The mechanism of this process is not known. Since the interval  $L_R$  runs its course between the instant of stimulation, at which the excitatory events in the muscle fiber membrane are set in motion, and the instant indicating the earliest change in mechanical state of the contractile system, we may conclude that the LR induction process includes all those reactions which link the action of the stimulus to the initial response of the contractile material—i.e. at least a portion of what may be called excitation-contraction coupling. As to the various chemical modifications caused by IAA that may account for the slowing of the LR induction process, it is possible to exclude the rise in pH. It has been shown (7) that a mere rise in pH of a normal muscle causes, in fact, a reduction in  $L_R$  and thus a speeding of the process in question. We are thus left with all the other alterations due to IAA as possible causative agents for the observed increase in  $L_R$  and there is no way at present of determining with certainty which of these is involved. We shall, however, return to this point later with the suggestion that the reduction in the ATP content of the muscle may be of significance.

We next consider the behavior of the tension latency,  $L$ . This parameter in the IAA post-tetanus muscle, after the immediate decrease, remains essentially unchanged for about 2 minutes, then decreases still more during the next 7 minutes after which it rises slightly over the course of the remainder (20 minutes) of the post-tetanic period. The general picture is one of a hastening of the onset of tension. This behavior is even more strikingly evident if, instead of considering changes in  $L$ , which includes the time of  $L_R$  and its underlying LR induction process, we analyze the variations in the interval  $L-L_R$ , thus restricting the time to that which embraces the at least somewhat parallel LR, and tension processes. Figure 5 presents the average percentage changes in  $L-L_R$  for both the normal and IAA muscles. In the normal case, this interval alters somewhat as does  $L$ . Under the influence of IAA

there is a greater immediate diminution that is maintained constant for about 3 minutes after which it then decreases markedly during the remainder of the post-tetanic period. This result may be further indicated by the average absolute values of  $L-L_R$ ; before tetanus this interval lasts 1.36 msec.; immediately following the activity its value is 0.98 msec. and at the end of the 30-minute post-tetanic period it is reduced to only 0.64 msec. Hence, after activity the tension induction process in the IAA muscle operates, in general, at an increasing rate. Now, evidently in consequence of the deleterious metabolic effects of IAA, there are obviously harmful effects on the mechanical behavior—the rigor development and the decrease in tension output. It might therefore have been expected that the tension induction process would also be adversely affected and would thus be slowed. But to the contrary, it is speeded up, despite whatever harmful changes may have been suffered by the muscle.

In the earlier paper on the effect of activity in the normal muscle it was possible to demonstrate that the changes in  $L$ , and more specifically of  $L-L_R$ , could be correlated with the activity-induced pH changes, i.e. the higher the pH, the smaller these time intervals. The present results are generally in accord with this correlation. For in the IAA muscle the irreversible pH increases engendered by activity are accompanied by irreversible decreases in  $L$  and  $L-L_R$ . In the previous study it was furthermore hypothesized that the tension induction process is mechanochemically represented by the formation of an enzyme-substrate complex between myosin-ATPase and ATP and that the rapidity with which this complex disintegrates, as ATP is hydrolyzed and the energy of its first phosphate bond is taken up by the contractile material, determines the rate of development of tension and thus the duration of the tension induction process as indicated by  $L-L_R$ . The basis for this hypothesis was established by showing first, that of all the mechanochemically relevant reactions known to occur in muscle, the hydrolysis of ATP, not only as such but also under the action of myosin-ATPase, is the only one that is speeded up by increase in pH, and second, that this reaction also possesses other well known properties (it is the earliest of the known metabolic changes occurring in activated muscle, it involves a great energy release due to the splitting of its energy-rich phosphate bonds, and it is the specific substrate of the contractile enzyme, myosin-ATPase) which all indicate that it is fundamentally concerned with the process of directly supplying energy to the contractile material. In so far as the IAA muscles of our experiments develop their characteristic pH changes, the above hypothesis serves to explain the observed behavior of the tension induction process. For the large and irreversible alkalization of the muscle would cause ATP to break down at a corresponding high rate throughout the post-tetanic period and thus hasten the tension induction process and consequently, in accordance with the actual records, reduce the time interval  $L-L_R$ .

Although this interpretation is reasonable, it is pertinent to raise several questions concerning it. In the first place, we have assumed (2, 7) that the ATPase of our hypothesis is the one originally described by Engelhardt (11) which has been shown to have a maximum activity at pH 9.0 (12), although recently Kielley and Meyerhof (13) have demonstrated that if this myosin-ATPase operates as part of



a myosin-actin (4:3) complex, which is somewhat like the form in which it may exist in live muscle, the optimum is shifted to about pH 7.6. Also, since the initial proposal of our hypothesis, another muscle ATPase has been discovered with a maximum of activity that has been stated to be at pH 7.4 in one publication (14) and is indicated to be at about pH 7.0 in another (13). It might be thought, since these pH optima are so low, that the increased alkalinity in activated IAA muscles, if it exceeded pH 7.4, say, would according to our hypothesis result in a diminished rate of ATP hydrolysis and hence in an increase in the interval  $L-L_R$ . However, it has been shown by Fenn and Maurer (15) that the intracellular pH of normal frog skeletal muscle is 6.9 even though it is in equilibrium with a Ringer's solution of pH 7.34. In our experiments the external medium had a pH of 7.1, and therefore the intracellular pH of at least the normal rested muscles must have been near to pH 6.9. As for the IAA resting muscles, it must be recalled that these were oxygenated, for under this condition, despite the inhibition of glycolysis, little change in the ATP or CP content and hence of pH occurs as long as the muscle is at rest. We may therefore infer that even in the case of the treated muscles, the intracellular medium before activity was also at about pH 6.9. Now the results of Dubuissou and Schultz (5) show that IAA-activated muscles develop at maximum an alkaline shift of the order of 0.1 pH unit. Hence it does not seem likely that our activated IAA muscles could have reduced their hydrogen-ion concentration enough to bring into play the diminished activity of the ATPases in question which appears at pH's above their optima.

The question may also be raised as to whether the new ATPase plays any part in our proposed mechanism. This enzyme is obtained in water extracts of muscle, while the Engelhardt ATPase, although it may be separable from myosin (16), is extracted from muscle along with myosin and hence must be strongly bound to it within the intact fiber. This may signify that in the live muscle, it is this strongly adsorbed ATPase that serves to catalyze the hydrolysis of ATP as involved in our mechanism. Since the optimum for this enzyme is, at a minimum, at 7.6, it is all the more likely that the pH increases of our activated muscles remain in a range such that corresponding increases in ATPase activity are engendered. Present evidence, however, does not permit us definitely to exclude the participation in muscular activity of the new Meyerhof ATPase.

We now consider the significance for our results of the interesting finding of Polis and Meyerhof (16) that IAA in concentrations of the order of  $10^{-4}$ – $10^{-3}$  M activates myosin-ATPase by as much as 30–45 per cent. Our 1–80,000 dilution of IAA corresponds to  $0.68 \times 10^{-4}$  M, and at this concentration myosin-ATPase is activated about 25 per cent. Hence we infer that in our treated muscles the speeding up of ATP hydrolysis is not only a pH effect, but also a consequence of this activation by IAA. Indeed, this may account for the fact that the poisoned muscles developed immediately after tetanus a greater reduction in  $L-L_R$  (30%, fig. 5) than the normal controls (25%).

A third question is the large diminution of  $L-L_R$  in the last 27 minutes of the IAA post-tetanus period. There are no data indicating a progressive increase in muscular pH during this interval, and hence we cannot attempt a correlation here

between pH and  $L-L_R$  changes. But the following may be suggested in explanation of the decrease in  $L-L_R$ . It is known that the resynthesis of ATP in muscle depends on the presence of CP (e.g. adenosinediphosphate +  $CP \rightleftharpoons ATP + \text{creatine}$ ). It would seem probable, as the store of CP is reduced and the tendency for resynthesis of ATP is thus diminished, that conditions would then be more favorable for the hydrolysis of ATP. If this were true, then it would account for the observed shortening of the interval  $L-L_R$ . Although our hypothesis postulates that the speed of hydrolysis of ATP determines the duration of  $L-L_R$ , it must be noted that the concentration of this substance in the activated IAA muscle continuously decreases in parallel with the increase in rigor, i.e. during the last 28 minutes of the post-tetanic period of our studies, when  $L-L_R$  is decreasing, and when, by assumption, the rate of ATP breakdown is increasing. To resolve this seeming contradiction it must be noted that the *amount* of ATP at any moment will determine the potential of its activity, but not the intrinsic rate of its decomposition. Thus, the decreases in R and T may be evidence of the effect of reduction in ATP concentration, i.e. of the potential of ATP in causing actual tension outputs. But this may be independent of the velocity constant of hydrolysis of ATP—i.e. it may yet be true that the various conditions we have discussed will act to increase this reaction rate and thus speed up the process by which actual tension output is made possible.

The fact that the ATP content is being irreversibly reduced during the last 25 minutes of the IAA post-tetanic period may, however, account for the observed progressive increase in  $L_R$  that occurs during this time (fig. 3). In earlier papers (1, 2, 7) it has been suggested that the interval  $L_R$  involves, among other possible events, the formation of activated ATPase which then combines with ATP in the enzyme-substrate complex form postulated to account for the latency relaxation and its associated tension-induction process. It seems reasonable to infer that when the ATP content of the muscle is markedly decreased this would delay the formation of the complex and thus cause the observed increase in the interval  $L_R$ . In the normal muscle there is no irreversible breakdown of ATP and this would account for the constancy of  $L_R$  throughout the 30-minute post-tetanic interval. It has been noted, furthermore, that the exercised IAA muscles rapidly approach death following the 30-minute post-tetanic rest and that this is indicated, in addition to the pronounced rigor, by further reductions in R and T and large increases in the values of the time parameters  $L_R$  and L. Evidently at this time the reduction in ATP becomes so great that the entire mechanism of initiating a response is impaired and this is reflected in the moribund latency and tension-output changes we have recorded.

Thus, our results are consistent with the implications of the hypothesis that ATP breakdown occurs during the latter portion of the latent period. This suggests that the energization or activation of contraction depends on activity coupling of myosin-ATPase and its substrate (e.g. 7, 17). But, since the present work includes no data of the post-contractile relaxation period, the possibility of an additional recovery coupling mechanism is not excluded. Quite apart from the problem of the temporal localization of this coupling, claims have been advanced (19, 20) that the decomposition of ATP by myosin-ATPase cannot have the energetic significance at-

tributed to it since the rate of phosphate turnover by extracted actomyosin in solution is far less than that resulting from contractile activity of living muscle. It is very doubtful, however, that the *in vitro* determination of the enzymatic activity represents the optimal potency for cellular behavior, e.g. the living physical state of myosin ATPase, though not definitely known, must be very different from its condition in solution. Furthermore, the great importance given to the roles of the calcium and magnesium ions in the activation and the inhibition of the enzyme in solution, has at present little significance for the point in question since the striational compartmentalization, especially during activity, of these ions and that of the enzyme itself is not known.

Our own conception of the energization of contraction, however, is admittedly dependent on only a limited number of results, which for the most part deal with latent period phenomena. Even with respect to latency behavior our work does not fully explain the changes in the depth of the latency relaxation in the twitch responses following a tetanus. It is notable, however, that this parameter in both normal and IAA muscle is smaller, the less is the interval  $L-L_R$ . Since a reduction in  $L-L_R$  indicates an increase in rate of tension onset, the associated decrease in  $R$  may be the resultant of a normally developing latency relaxation that is cut short by the more rapidly initiated tension output. More work needs to be done on this latency feature, probably in relation to the kinetics of the latent period mechanical changes (see 7). Moreover, additional tests must be made of our view that certain of the activity induced latency alterations are consequences of pH alterations. In particular it will be of interest to study these modifications as a function of the initial pH of the muscle since it has been shown (e.g. 5, 20) that as the pH is lowered from about 7.0 to 6.0, the pH increases resulting from activity are greatly enlarged and this, provided our inference is correct, should lead to correspondingly larger relative diminutions in  $L$  and  $L-L_R$ . Finally, experimental tests of our proposed mechanism must be performed with respect to other periods of a muscular contraction, and especially is it necessary to determine the relation of our observed latency changes to similarly conditioned alterations of the rate of development of tension in the contraction period. Researches of this sort are planned or in progress and the results will be published elsewhere.

#### SUMMARY

Studies have been made in muscles treated with iodoacetic acid (1-80,000) of the effect of activity of various types on the subsequent mechanical behavior, especially of the latent period in isometric twitches. The IAA effects are most clearly demonstrated in muscles after subjection to a short tetanus, e.g. of 3-second duration.

Immediately following a tetanus, the IAA muscles exhibit latency changes (reduction in latent-period duration and depth of the latency relaxation) which are more pronounced than those found in normal muscles. During the post-tetanic period of 30 minutes the alterations of the IAA muscles are irreversible (or they become larger), while in the normal tissue they are reversible. Although for the normal muscle the latency for the latency relaxation is unchanged by the activity, it is increased in the IAA muscle during the latter 25 minutes of the post-tetanic rest period.

These features of the post-tetanic behavior of the poisoned muscles are ascribed to the irreversible increase in pH and the irreversible depletion of adenosinetriphosphate characteristic of IAA muscles following activity, and to the activation of myosin-ATPase due to IAA in low concentration. This interpretation is shown to be consistent with the hypothesis that the energization of contraction is dependent on the breakdown of ATP by myosin-ATPase initiated during the latter part of the latent period.

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## FACTORS RELATING TO HEART SIZE IN THE INTACT ANIMAL<sup>1</sup>

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AUGUSTA, GEORGIA

A METHOD has been presented for measuring the size of the dog's heart from the x-ray shadow (1). Observations made then indicated a very large variation in the size of the heart within the chest of the intact animal for the heart in the same animal might vary from 600 cc/m.<sup>2</sup> after a large dose of epinephrine to 200 cc/m.<sup>2</sup> after severe hemorrhage.

The empty heart varies in dogs of different breeds and different histories (2). Part of this variation is anatomical, part physiological. To delineate the factors causing physiological change in heart size, we have put all hearts on a more comparable base line by plotting the heart size on a scale that begins at zero for the smallest heart which the animal exhibited after severe hemorrhage. All of the figures on the abscissa scale of our plots, then, represent blood which is stored in the heart and which can be ejected under conditions of hemorrhage. This is called, for convenience, net heart size or cardiac blood volume. It is given numerical values adjusted to body size on a square meter basis.

Discussed here are a series of experiments in which net heart size was observed under many different conditions. Some dogs were subjected to hemorrhage with or without vagotomy. Others were infused with epinephrine during control periods and during a stepwise hemorrhage. Others were killed by progressive bleeding after producing a hypertension by carotid sinus removal and vagotomy. In several dogs, observations were made when the venous return was hindered by inflating a balloon placed by catheter in the thoracic inferior vena cava. Acute responses to sympathetic and parasympathetic drugs were also observed.

In addition to the measurements of net heart size, aortic pressure pulses were recorded optically. From these, the stroke volume, resistance, mean systolic pressure, cardiac work, and filling time were obtained (3). In several dogs the pressure in the superior vena cava near the heart was recorded by means of a differential manometer which was balanced against intrathoracic pressure (4) and which thus measured cardiac filling pressure. The record was read at the height of the A wave, since this probably comes closest to measuring the pressure distending the ventricle before systole. The manometer, therefore, was set to read zero at the level of the apex beat. The heart size, as measured from the x-ray P-A silhouette, is determined largely by the diastolic size of the ventricles (1). By subtracting the minimal heart size and plotting the remainder we were dealing largely with changes in the blood volume contained in the two ventricles.

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The net cardiac blood volume is controlled by factors which are related to diastolic filling and to systolic emptying. Possible factors affecting diastolic filling are: 1) diastolic duration and the time course of ventricular relaxation, and 2) filling pressure. Systolic emptying leaves varying amounts of residual blood in the ventricles and this is added to the inflowing blood to determine diastolic size. Possible factors related to the amount of residual blood are: 1) mean systolic pressure, 2) stroke volume, and 3) cardiac work. In the intact animal all of these factors may act simultaneously. It is our purpose to evaluate their relative importance.

*Diastolic Time* (fig. 1). The duration of diastole correlates positively but with considerable scatter with cardiac blood volume. Study of figure 1 shows that the different experimental procedures have no dominant effect upon heart size other than through their effect upon heart rate. Points representing epinephrine infusion, buffer

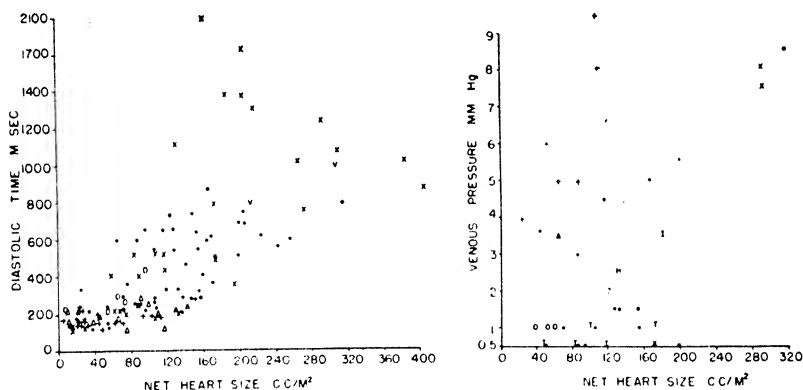


Fig. 1 (left). RELATION OF DIASTOLIC TIME to intracardiac blood volume above minimal. ● = Normal and bled dogs; + = vagotomized and bled dogs; △ = dogs with sectioned buffer nerves and bled; A = effect of mecholyl; I = effect of Isuprel; T = transfusion after hemorrhage; X = epinephrine infusion in normal and bled dogs; \* = epinephrine infusion in vagotomized and bled dogs; V = vagus stimulation.

Fig. 2 (right). RELATION OF HEART SIZE to venous pressure. Symbols as in figure 1.

nerve section, vagotomy, and simple hemorrhage are scattered with very little difference in their relation to a line which indicates that on the average there is an increased heart size with increasing filling time.

At very short diastolic time, i.e. between 125 and 250 msec. there is a range of net heart size from 10 to 140 cc. The larger of these hearts must have been incompletely emptied during systole or very rapidly filled during diastole. The first of these possibilities is the more probable, because, as will be seen below, in those fast pulses, when the venous pressure is relatively high, and where conditions are such (low arterial pressure) as should lead to complete systolic emptying, the hearts are not large. Many of the larger of the fast hearts were working against a high arterial pressure (buffer nerve section, vagotomy, epinephrine infusion) and hence might well owe their diastolic size largely to an increased residual blood.

*Filling Pressure.* With heart rate, myocardial reserve and arterial pressure constant, Starling (5, 6) found in the heart-lung preparation, that an increase in

venous pressure led to an increase in diastolic size. In the intact animal this tendency is partly obscured by other variables.

In figure 2 we have plotted, in relation to heart size, the difference between intrathoracic pressure and right auricular pressure at the time the auricles contracted. We have measured pressures from the level of the apex beat at zero, and have used only the few observations in which our venous pressure records seemed to be without artifacts.

There are three trends in the relationship between venous pressure and heart size: 1) Low pressure and varying heart size, 2) varying pressure and varying heart size, and 3) varying pressure and small heart size.

In the group with very low venous pressure the heart size varied with diastolic time, the fast heart being small and the slower heart being large. The slow hearts enlarged despite the fact that the venous pressure curve remained low and flat during diastole.

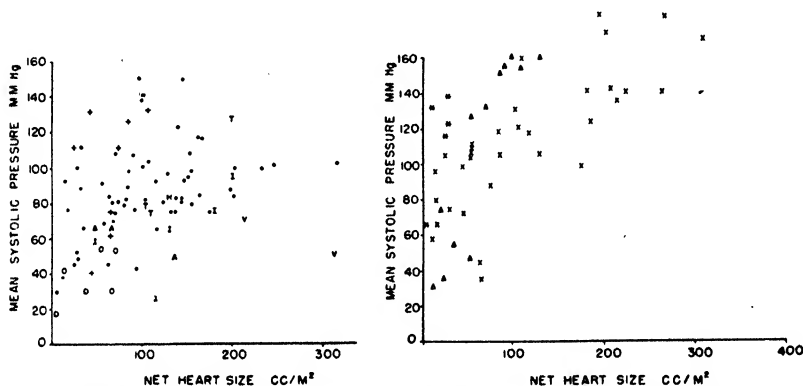


Fig. 3. RELATION OF HEART SIZE to mean systolic pressure. Symbols as in figure 1.

The second group also included fast and slow hearts. In this group, not only were the slow hearts larger, but the venous pressure was greater. The venous pressure curve showed large pulsations with auricular and ventricular contraction and rose steeply during diastole.

The third group included only fast hearts. The hearts remained small even though the venous pressure rose steeply with auricular contraction to levels 4 to 8 mm. Hg above the level of the apex beat.

*Mean Systolic Pressure* (fig. 3). If mean systolic pressure were the only variable, it would follow from the work of Starling (5, 6) that the greater the pressure against which the heart works, the greater the amount of residual blood and the larger the diastolic size of the heart. This would also be congruent with the idea that energy release is proportional to diastolic size.

The data indicate that there is a tendency in the intact animal for the heart to be larger as the mean systolic pressure rises. This is probably due in part to the direct effect of aortic pressure in hindering ejection and in part due to the slowing of the heart in reflex response to the rise in pressure. The dominance of heart rate

in setting diastolic size is shown by the fact that when the heart is slowed by vagus stimulation it is large in spite of the relatively low mean systolic pressure and cardiac work.

Animals whose blood pressure was at the control level or lowered by hemorrhage, acetylcholine (rapid heart phase), isuprel, or occlusion of the inferior vena cava, or which had suffered both hemorrhage and reinfusion, showed a correlation of pressure fall with a gradual diminution of heart size until the cardiac blood volume became small (75 cc. or less). These small hearts might sustain mean systolic pressure anywhere between 25 and 75 mm. Hg.

Infusion of epinephrine caused the mean systolic pressure to be very high, and in the control dogs the heart was very slow and large. As these dogs were bled their hearts accelerated and became smaller with mean systolic pressure falling only slightly. After further hemorrhage the cardiac blood volume was reduced to 100 cc. or less as the mean pressure fell further. Net heart size below 50 cc. might be correlated with mean systolic pressures ranging from 30 to 130 mm. Hg. In dogs receiv-

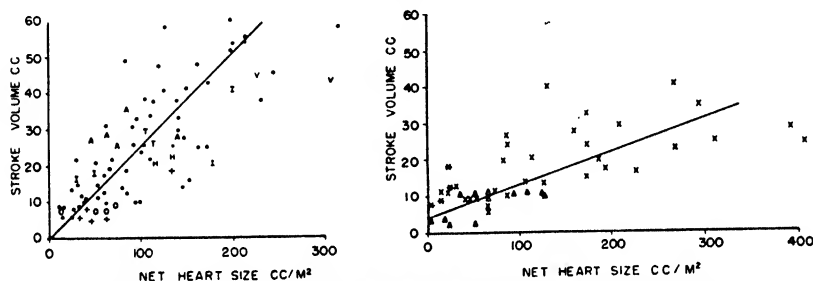


Fig. 4. RELATION OF HEART SIZE TO STROKE VOLUME. Symbols as in figure 1.

ing epinephrine infusion small hearts maintained higher pressure levels when the vagi were cut than when they were intact. This is also true of dogs whose carotid sinus nerves and vagi had been cut but which did not receive infusions. All of these hearts were beating rapidly and it is only when there was reflex slowing that the epinephrine stimulated hearts dilated.

*Stroke Volume* (fig. 4). Starling found in the heart-lung preparation, that when other variables were held constant, the heart size was correlated with the stroke volume. In the intact animals, also, we find that the stroke volume is directly correlated, but with considerable scatter, with net heart size. There are two groups, however. The dogs subjected to epinephrine infusions, or buffer nerve section, showed large hearts in relation to stroke volume, while the dogs subjected to other drugs, hemorrhage, transfusion, etc. showed a smaller increase in net heart size as the stroke volume increased.

In the previous section it was seen that epinephrine infusion caused the heart to eject against much higher systolic pressures than normal, and that if the heart was not reflexly slowed, it did so at a relatively small diastolic size. Here it appears that this high resistance to ejection restricts the stroke volume so that the heart ejects a much smaller part of its diastolic blood volume than does a normal heart. This tendency is exaggerated when the heart is reflexly slowed.



**Cardiac Work Per Beat** (fig. 5). Just as increases in stroke volume and arterial pressure tend to parallel increase in the size of the heart in the controlled heart-lung preparation, so also is there an increase in heart size with work, which is the product of the two. In the intact animal, cardiac work (mean systolic pressure times stroke volume) also shows an increase which correlates with an increase in heart size.

In this relationship there were three groups: *a*) dogs receiving epinephrine infusion in which the heart reflexly slowed and became large relative to work done; *b*) vagotomized dogs that had an epinephrine infusion, whose hearts did not slow and were small in relation to the work done, and *c*) the control, hemorrhage and miscellaneous dogs in which the relationship was intermediate. Dogs with buffer nerve section fell in the miscellaneous-control group in relation to work though they were more like the epinephrine dogs in the correlation of their heart size with mean systolic pressure and stroke volume.

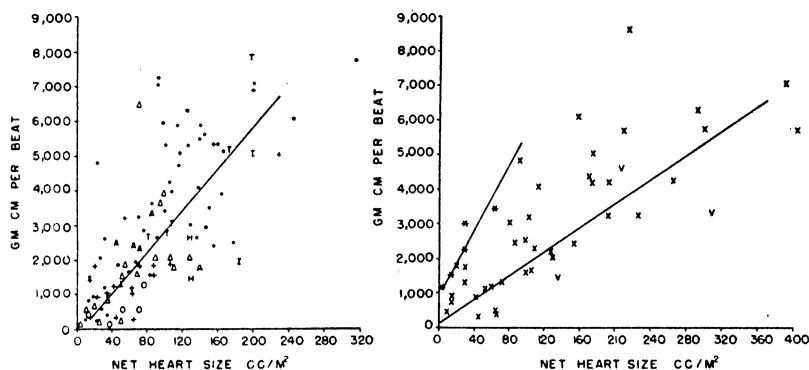


Fig. 5. RELATION OF HEART SIZE to work per beat. Symbols as in figure 1.

**Cardiac Work Per Minute.** In the Starling experiments heart rate was for the most part held constant so that there was no difference between output and work per minute or per beat in its relation to heart size. In the intact animals of the control and miscellaneous group there was a rough tendency for large hearts to be correlated with large work figures. The relationship was quite different in the dogs infused with epinephrine. When there was great reflex slowing, the hearts were large and the work was small. As the animals were bled, the heart speeded up and the work continued constant at first, and then increased while the heart size continued to diminish, so that very small hearts might show very large amounts of work per minute. Dogs with vagi or buffer nerves severed showed large work-per-minute figures in relation to their small hearts.

#### DISCUSSION

The classical work which stemmed from Starling's laboratory showed that the diastolic size of the ventricles determined their energy release. Thus if rate and output were held constant, diastolic size increased with arterial pressure; if rate and pressure were held constant, diastolic size varied with venous inflow and hence

stroke volume. In general, the diastolic size was closely correlated with the oxygen consumption of the heart and with the work which it could do so long as the rate and physiological condition of the heart remained constant. Moreover, it has often been held—though it was denied by Starling—that the level of the venous pressure is an index to the relative diastolic size.

In studying the intact animal it is impossible to hold two or three variables constant and allow another to change. Partial correlation plots in which the heart size was corrected for variations in diastolic time showed all of the observations distributed on either side of the average in a rather wide scatter. When these points were plotted against mean systolic pressure, stroke volume or work per beat, no clearly significant tendency was seen for the heart to be large or to be small in relation to these variables. The tendency for the epinephrine infused dogs to have relatively high mean systolic pressures, small stroke volumes and diminished work shows in these plots as in the others. Tendencies for heart size to vary in a systematic manner with pressure, stroke volume or work which is seen in the plots uncorrected for diastolic time, nearly disappears when this correction is made.

In the intact animal the preponderant factor in the control of the heart size seems to be the diastolic filling time. Hence the correlation between the mean systolic pressure and diastolic size is largely set by the fact that when pressure is high, pulse rates are slow unless the natural regulation of the heart is interfered with by vagotomy, atropine or vagus stimulation. When these latter conditions obtain, there is no longer the usual correlation between heart size and mean systolic pressure. In addition to this, is the fact that when the arterial pressure is high the heart empties itself less completely. Since some hearts are in worse physiological condition than others, the amount of residual blood inferred from the diastolic size bears no orderly relation to mean systolic pressure when allowance is made for the effect of filling time. Furthermore, when the heart is slowed, increases in stroke volume, and increases in the work done per beat are the immediate results of the fact that more time is allowed between beats to fill the heart and to make blood available for the next beat. The heart rate is the physiological variable that is most immediately under control and can be said to play the most fundamental and causative role in setting diastolic size.

The detailed contour of the venous pulse differs with the height of the venous pressure, because, as Opdyke (7) and his associates have shown, the auricle (and great veins) are highly distensible at low pressures and much less so at higher pressures. Thus at high venous pressures the contraction of the auricle causes a manifest increase in venous pressure. Furthermore, the pressure rises steeply, and shows adventitious waves during diastole. At low venous pressures auricular contraction and diastolic filling of the ventricle occur with a nearly flat venous pressure curve.

It is commonly held that when the venous pressure is low, filling is less and the heart is small. This concept is an over-simplification. In the heart-lung preparation, where other variables can be controlled, the heart does behave in this manner. The heart in the intact animal, on the other hand, acts in response to all variables simultaneously and may enlarge in response to increased diastolic time, increased filling pressure or increased residual blood—one or two at a time or all together.

When the venous return is large and the heart rate is slow, the venous pressure

shows a steady increase during diastole and a good peak of 8 to 10 mm. Hg., resulting from auricular contraction. These are the accompaniment of filling and distention of the ventricle and are congruent with the fact that these hearts will vary in size with filling pressure and with diastolic time because the two vary together.

After atropine or vagotomy, the heart rate is very rapid, and the venous pressure may reach high levels (4-8 mm. Hg. with auricular contraction) while the hearts remain small. Such pressures may be as high or higher than those seen in large slow hearts and if unopposed by back pressure from slow ventricular relaxation would, we think, have filled the heart to a large size even in a short diastole. Our tentative idea is that inflow into the ventricle must be opposed by resistance of the heart itself to filling. We think of the intrinsic rate of relaxation of the ventricular walls as playing a predominant role in maintaining a pressure in the ventricle nearly as high as that in the auricle, and thus controlling the rate of ventricular filling. Published records (8) show that pressures during diastole are nearly identical in auricle and ventricle, and that the volume curve during early diastole shows no reflection of auricular pressure oscillations (9).

The intrinsic control of ventricular filling by the process of myocardial relaxation is also shown in hearts with low filling pressure. These hearts are large if diastolic time is long, and small if diastolic time is short. The venous pressure pulse is flat with a small rise caused by auricular contraction. This is in part due to the large distensibility of the auricular walls at low pressures and in part to the fact that the enlarging ventricle accepts blood as fast as it returns with none left over to distend the veins and raise the pressure.

How long a time the process of relaxation of the ventricle takes cannot be told. With rapid heart rates it seems to us to be probably the dominant factor in setting the pattern of diastolic inflow. With slower rates, active relaxation may merge into passive distention if the venous pressure is relatively high.

It is not impossible on the other hand that when venous pressure is low, the large size of slow hearts may in part be accounted for by an aspirating action of the enlarging ventricle. That the ventricle is capable of such an aspirating action can easily be demonstrated. If tubes are tied into the natural orifices of the ventricle, compressing the heart will cause the ventricles to expel their contents of fluid. Release of the compression will cause the aspiration of fluid to a height of 5 to 10 cm. of saline. That this factor plays a role in the naturally relaxing ventricle is not proven though it fits well with the increased filling of slow hearts whose venous pressure has not increased.

#### SUMMARY

Heart size in intact animals is directly correlated with time of diastole, mean systolic pressure, stroke volume, and work per beat. Physiological and technical variables produce a wide scatter.

Epinephrine infusion produces a slow large heart with high mean systolic pressure, but with diminished stroke volume and work per beat as compared to undrugged dogs. Vagotomy, with epinephrine infusion or with carotid sinus removal, gives a rapid small heart with high mean systolic pressures and diminished stroke

volume and work per beat as compared to untreated dogs. These differences persist with bleeding.

At high normal venous pressures, reflex changes in diastolic time are paralleled by changes in venous pressure and in heart size. At very low venous pressures the heart may be large or small, depending on filling time. At rapid rates, the heart is small and the venous pressure may be high or low depending on venous return.

The most important factor in fixing the diastolic size of the heart, in the intact animal, is the filling time (heart rate).

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# CARDIAC OUTPUT IN THE RAT AT NORMAL AND AT HIGH ALTITUDES AND ITS RELATIONSHIP TO GAS EMBOLISM<sup>1</sup>

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THE principle proposed by Fick in 1870, which correlates the oxygen and carbon dioxide content of the blood and the gaseous exchange in the lungs, with cardiac output, represents the real beginning of the experimental determination of cardiac output. The effectiveness of any method based on the Fick principle depends upon the development of techniques for obtaining reliable samples of mixed venous blood. In general, the methods used have included cardiac puncture or introduction of a flexible catheter through a vein into the heart.

Cardiac output has been determined in a number of animals such as dogs, goats, rabbits and humans. However, since it has been shown that the rat is suitable for research in physiology as well as for student use as a laboratory animal (1), it seemed desirable to study the adaptability of the determination of cardiac output to the rat. The rat is anatomically suitable for this purpose since it is possible to introduce a metal cannula directly into the right auricle of the heart through the right jugular vein (fig. 1). This makes it possible to obtain highly reliable samples of mixed venous blood.

In a previous study on high-altitude physiology (2), it appeared that some relationship may exist between the cardiac output and the occurrence of gas embolism. Thus, the method for the determination of cardiac output was further developed so that cardiac output could be determined at simulated high altitudes and an attempt was made to correlate the cardiac output at high altitudes with the development of gas bubbles in the heart and circulatory system.

## EXPERIMENTAL PROCEDURE

Young adult rats weighing 180 to 200 gm. of the University of Denver strain were used. They were anesthetized by subcutaneous injection with sodium pentobarbital in a dose of 37.5 mg/kg. body weight. The trachea was cannulated to insure a free passage of air into the lungs, and the left carotid artery was cannulated in order to obtain arterial blood samples. A small brass cannula, about one mm. outside diameter, was introduced into the right external jugular vein, through the superior vena cava and into the right auricle. The proper distance for introduction of the cannula was marked by previous measurement in rat cadavers.

The apparatus used for the determination of cardiac output at Denver and

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simulated high altitudes is illustrated in figure 2. The chamber was constructed from cast brass and had a lucite window sealed in the cover. It consisted of two compartments, one for the rat and a smaller one which could be maintained at constant pressure during the period oxygen consumption was being determined. The rat was fastened in a rack which also served as a support for the wicks soaked in 10 per cent sodium hydroxide used to absorb the carbon dioxide produced by the animal. The carotid and cardiac cannulae were attached to small brass tubes which extended through the end of the chamber and one-ml. syringes were attached to the outside ends of the tubes. The chamber was placed in a constant temperature bath maintained at 15° C.

When the chamber had reached constant temperature, the tube connecting the two compartments, the air inlet tube and the tube to the vacuum pump and altitude manometer were closed with pinch clamps. A drop in pressure resulted in the rat compartment due to consumption of oxygen by the animal; this in turn caused the level of the manometer fluid to rise. Oxygen was admitted from a gas burette so as to maintain the manometer liquid at a constant level. At the same time, 0.2-ml. samples of arterial and mixed venous blood were drawn into the one-ml. syringes. The length of time during which oxygen consumption

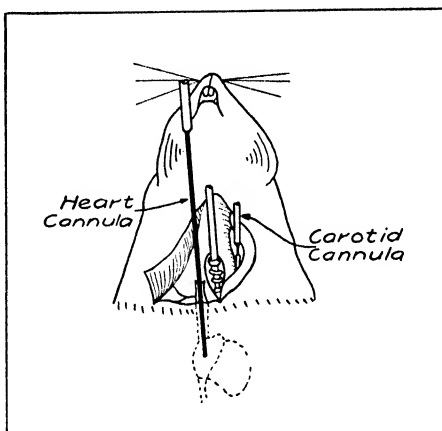


Fig. 1

was being determined was noted by means of a stopwatch. At the end of the test, all clamps were removed and the pressure in both chambers was decreased at a constant rate equivalent to 2000 feet/minute until the desired altitude was reached. Air was bled through the chamber during the ascent at a rate of 4 l/minute. After constant temperature had again been reached, blood samples were obtained and oxygen consumption determined as above. The oxygen content of the arterial and venous blood samples was determined by the micro-method developed by Roughton and Scholander (3); cardiac output was calculated according to the following relationship based on the Fick principle.

$$\text{Cardiac output (ml./min.)} = \frac{\text{O}_2 \text{ consumption (ml./min.)}}{\text{Arteriovenous O}_2 \text{ diff. (ml./100 ml. blood)}} \times 100$$

In one group of animals, cardiac output was determined at 5280 (Denver's altitude), 20,000, 30,000 and 40,000 ft. but since this necessitated the removal of a rather large percentage of the total blood volume, cardiac output was determined only at 5280 and 40,000 ft. in a second group used for the correlation of cardiac output at high altitude with the occurrence of gas emboli.

The vessels and organs examined for gas emboli were the femoral vein, the

portal vein, the inferior vena cava, the mesenteric veins and the heart. Examination of the lungs failed to give significant information; emboli were found only in severe cases where there was a large amount of gas in all vessels. It should be pointed out that the extent to which emboli formed varied greatly from animal to animal.

### RESULTS

*Cardiac Output at Denver's Altitude.* The cardiac output was determined on 52 rats at 5280 ft.; the mean was found to be 46.5 ml/minute. The standard deviation for the group was 15.6 and the standard error 2.2 ml/minute.

*Change in Cardiac Output with Change in Altitude and its Relationship to Gas Embolism.* In most animals, it was found that an increase in altitude produced a decrease in cardiac output. Since the arterial oxygen saturation is 10 to 20 per cent

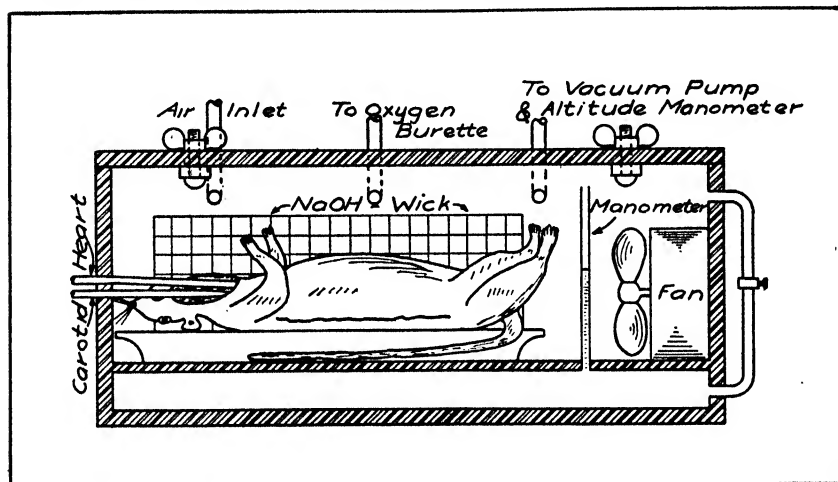


Fig. 2

in animals at an altitude of 40,000 feet (4), it is quite probable that this had considerable influence on the decrease in cardiac output. Further, there appears to be some relationship between the magnitude of decrease in cardiac output and the formation of gas bubbles in the circulatory system. The mean percentage decrease in cardiac output for 18 animals at 40,000 ft. developing no emboli was 25 per cent, S. D. 19.7 per cent while in 12 animals in which emboli did form, the decrease was 67 per cent with a S. D. of 20 per cent.

A comparison of the mean cardiac output at high altitudes in rats developing no emboli with the mean cardiac output in rats having emboli indicates that a difference exists (table 1) and, when the values for the two groups are analyzed statistically, the difference is found to be significant. There appears to be a critical value for cardiac output of about 20 ml/minute; rats having a cardiac output less than this value at high altitude, developed gas embolism while those having a cardiac output greater than this value developed no emboli. This relationship between cardiac output and

the occurrence of gas embolism may be explained on the basis of the rate at which small nuclei grow until bubbles, which may be considered emboli, are formed. If the cardiac output is high (circulation time short), it is probable that the gas nuclei will pass on into the lungs where they will be lost before they are large enough to cause trouble. On the other hand, when the cardiac output is low and thus the circulation time rather long, the nuclei may have time to build up into large enough bubbles that they would not be carried along by the blood so could not diffuse from the lungs but would continue to coalesce until they would be of sufficient size to prevent efficient circulation.

It is possible that the decrease in cardiac output may be the result of the formation of emboli rather than the cause; however, since at least half of the animals in which emboli were found had no gas in any part of the heart, and, since it was pos-

TABLE 1. CARDIAC OUTPUT IN RATS AT HIGH ALTITUDE

	18 ANIMALS WITHOUT AIR EMBOLISM ml/min.	12 ANIMALS WITH AIR EMBOLISM ml/min.
Mean.....	40.5	13.0
S.D.....	21.3	4.1
S.E.....	4.8	1.2

sible to prevent the occurrence of emboli in animals at high altitude by bleeding oxygen into the chamber instead of air, it is our belief that the low cardiac output probably contributes to the production of the emboli rather than the reverse. Further study will be necessary before this can be fully answered.

#### SUMMARY

A method has been developed for the determination of cardiac output in the rat at normal and high altitudes using the Fick principle. The cardiac output in the rat at Denver's altitude (5280 ft.) was found to be 46.5 ml/minute. The cardiac output decreases when an animal is taken from normal to high altitude; the relative magnitude of decrease appears to have some relationship to the development of gas embolism. A close correlation exists between cardiac output at high altitude and the occurrence of gas embolism.

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## EXCITABILITY OF THE MAMMALIAN VENTRICLE THROUGHOUT THE CARDIAC CYCLE

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THE concept that there is an excitability cycle of the heart containing an absolute refractory period, a relative refractory period and a somewhat longer interval of normal excitability had its origin in the work of Fontana, Bowditch, Kronecker, Marey and others (1). A period of supernormality following the relative refractory period was found to occur under certain conditions (2-5) and it has been suggested that there may be a subsequent subnormal period (6). Measurements both of the basal excitability and the phasic changes in excitability have been made on numerous occasions. The generally accepted conclusion is that recovery of excitability throughout the relative refractory period is a smoothly progressive process (7). Recent work (8, 9), however, has indicated otherwise. This report is an account of results obtained from a study of the excitability cycle of the mammalian ventricle by means of improved techniques.

### METHODS

The apparatus and techniques employed in the experiments on 52 dogs, contributing results to this paper were essentially the same as those described in previous communications (9, 10). The heart was exposed by a midline longitudinal incision through the chest wall. A pericardial cradle was constructed and electrodes were attached to the base of the right ventricle or to both ventricle and right auricle. Pairs of pick-up electrodes were placed on various regions of the right and left ventricles and employed as direct leads to the recording instruments. The sino-auricular node was crushed and the heart driven by stimuli applied to auricle or ventricle at a rate slightly above spontaneous nodal rhythm. Normally the heart was driven at rates between 100 and 200 beats per minute, the cycle lengths varying between 600 and 300 msec. A cycle of 400 msec. was most commonly used. With auricular drive separate pairs of auricular driving and ventricular test electrodes were employed. When the ventricle was driven and tested directly only three ventricular electrodes were used, one serving as a common anode for testing and driving

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stimuli. These were the standard procedures followed in this work. Other details of technique will be given in connection with the descriptions of results obtained.

### RESULTS

Several methods of recording results were employed and a variety of different but related phenomena were studied.

1. *Determination of Excitability by Means of Strength-Duration Curves.* Figure 1 shows the strength-duration curves obtained in one experiment by testing excitability at various intervals of the cycle. The intervals are designated as milliseconds after the driving stimulus by the figures on each curve. The results are typical of other experiments and show at least four phenomena of interest.

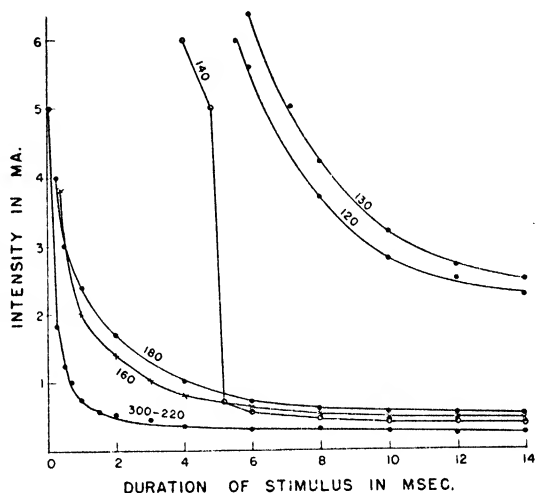


Fig. 1. FAMILY of strength-duration curves taken at indicated msec.-intervals after origin of beat showing jump (cf. curve at 140 msec. with curves taken earlier in the cycle, e.g. 120, 110) and dip phenomena (cf. curve at 160 msec. with curve at 170 msec.)

a) The shapes of the strength-duration curves and the relationships between them indicate that chronaxie determinations under conditions such as these are misleading. Actual calculations of the chronaxies in this series of experiments show that the chronaxie does not always reveal the state of excitability indicated by the position of the total strength-duration curve.

b) The strength-duration curves are of two types, making up a low- and high-intensity group. At certain intervals the long-duration stimuli give thresholds in the range of the low-intensity group, but with shorter-duration stimuli there is a jump to the high-intensity family. This is suggestive of the shifts from alpha to gamma curves obtained from isolated skeletal muscle by Rushton (11) due to a shift from indirect to direct stimulation of muscle fibers. It is thought, however, that this dichotomy shown in figure 1 is merely an expression of the fact that at certain intervals early in the cycle there is refractoriness or unresponsiveness to all durations of stimuli, at later intervals there is refractoriness to short durations and at all other times

refractoriness to none. This difference in sensitivity to different durations of stimulus shock is related to the production of persisting excitatory states and has bearing on the definition of the true boundaries of the refractory period, a matter which will be discussed later.

c) It is apparent that long-duration shocks stimulate and create effective excitatory states at much lower intensities than do the short-duration shocks. If, however, one calculates the total current-flow used in stimulating the heart muscle

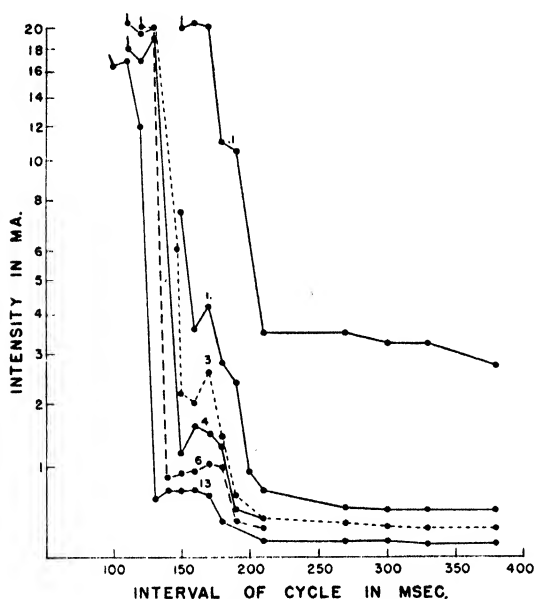


Fig. 2. CURVES obtained by determining threshold strengths of stimuli of various durations throughout cycle. Primary dip at 100- to 120- msec. interval; major dip at 150- msec.

msec., respectively, after driving stimulus) thus indicating a decreased threshold and increased excitability. This reversal of expected position occurs only in the relative refractory period and gives evidence of an asymmetry of the recovery process. Such fluctuations may take place at more than one interval of the cycle. They were clearly present in the majority of experiments in which strength-duration curves were plotted. It was realized that a different type of testing technique would permit clearer demonstration of these 'dips' or periods of relatively increased excitability in the refractory phase of the cycle. Consequently curves were obtained by testing with one duration of stimulus shock throughout the entire cycle. Various durations were employed in turn. Figure 2 gives a typical example of the results.

2. *Excitability Curves for Stimuli of Fixed Duration.* In curves expressing excitability changes obtained with stimuli of intermediate and long duration the asymmetries of the recovery process were more easily observed. Stimuli of long duration revealed one major late dip and one or rarely two earlier ones. These oscil-

at any one interval of the cycle, the short-duration shocks are found to be more effective in terms of micro-coulomb requirement. This difference in relative effectiveness may be explained by the greater degree of accommodation to stimuli of longer duration.

d) If recovery of excitability were a symmetrical, smoothly progressive phenomenon, one would expect strength-duration curves taken progressively earlier in the cycle to be above and to the right of curves obtained late in the cycle. It can be seen in figure 1 that at 160 and 120 msec. the curves were actually lower and more to the left in whole or in part than curves obtained later in the cycle (170 and 130

lations can be described in the following manner. Stimuli near maximal strength (20-30 ma.) often produced a response during a 10-msec. interval early in the cycle but failed to do so when placed a few milliseconds later, as though regression had occurred and the heart had again become absolutely refractory. Ten or twenty msec. still later in the cycle maximal stimuli again were effective indicating a second recovery of excitability. Characteristically, there was then a quick extensive recovery of ventricular excitability which brought the threshold almost to normal. Another recession then occurred which lasted for as long as 30 to 40 msec. before recovery processes again came into ascendancy and normal excitability was attained. The primary or height intensity 'dip' was demonstrable in the majority of experiments and the major or late low-intensity dip was present almost without exception.

TABLE 1. POSITION OF DIPS IN CYCLE

EXPER. NO.	CYCLE LENGTH	DURATION OF TEST SHOCK	END OF ABS. REF. PERIOD AFTER BEAT ORIGIN	TIME AND DURATION OF 'DIP' AFTER ORIGIN OF CYCLE	
				Primary	Major
	msec.		msec.	msec.	msec.
34	480	13	90	90-100	120-170
35	480	13	100	110-120	140-190
38	480	13	70	70-80	100-140
38	480	3	120		120-140
40	480	13	100	100-110	130-160
40	480	3	110	110-120	150-170
42	480	13	60	110-120	130-170
49	400	13	60	80-120	160-190
55	400	13	80	90-100	120-150
64	400	13	80	90-100	120-140
70	400	13	80	80-90	110-130
71	400	13	110	110-120	130-160
87	400	13	85	90-110	130-160
94	450	3	140		160-170
95	400	3	115		140-160
135	400	3	130	130-140	160-170

Stimuli of different durations revealed these same dips at the same positions in the cycle except when there were changes in the condition of the heart which prolonged or modified the duration of refractoriness and moved curves to right or left of their previous position. Curves obtained with very short-duration stimuli did not show as many oscillations as did the long-duration curves. This is probably due chiefly to the fact that the former were not all able to stimulate sufficiently early in the cycle to effectively 'enter' the areas of relative hyperexcitability. The major or late dip, however, has been revealed by all stimulus durations from 0.1 to 13.0 msec.

3. *Studies of Dip Phenomenon.* The commonly employed experimental procedure was to use three closely placed (5-10 mm. apart) electrodes for driving the heart and testing its recovery of excitability. It was thought that the dips might be artifacts of our procedures. Consequently we performed an extensive series of experiments which enabled us to reach the following conclusions:

a) As shown in table 1 the dips are present at specific intervals of the recovery

period. They are positioned with respect to the instant of initiation of the beat and the duration of the cycle. Other factors, to be discussed in later communications, may influence their position; but under uniform conditions the dips have a relative fixed and predictable locus in the recovery curve.

b) Dips can be obtained by various types of stimuli: induction shocks, condensor discharges, and various durations of rectangular pulses.

c) The fact that very short duration stimuli do not demonstrate the dips because they cannot produce an effect at the early dip intervals is also consistent with the idea that the dip is a physiological phenomenon of recovery and not an artefact created by driving or testing stimuli.

d) Occurrence of the dip phenomenon is not dependent on the use of a certain shape, size, form or type of electrode. It was obtained with platinum, chlorided silver, saline-wick, punctate, loop-shaped (3 mm. in diameter), bipolar, stigmatic monopolar and with electrodes making light contact with the surface or buried in the ventricular muscle wall. The stigmatic electrodes were paired with similar electrodes placed on chest or neck muscles.

e) That the dip is not created by the driving electrodes or by interaction between driving and testing electrodes follows from the fact that dips are obtainable when the heart is being driven either through auricular electrodes or by its S.A. or A.V. nodal pacemaker.

f) The arrangement and orientation of electrodes, however, is not without influence upon the results obtained from testing the excitability of the heart. Use of monopolar and bipolar stimulation gives different excitability curves as indicated below.

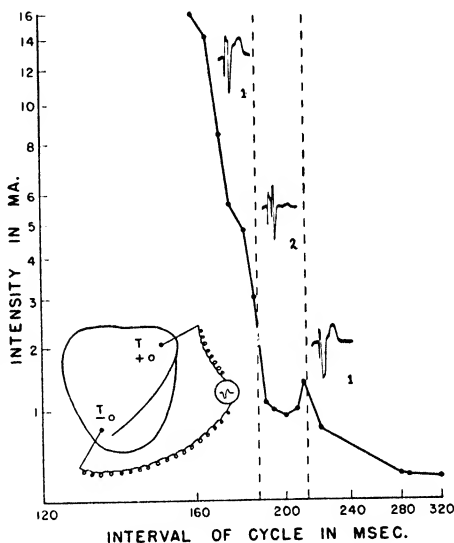
*Monopolar stimulation with stigmatic cathode.* This technique uniformly revealed an early or high-threshold dip within a few milliseconds of the end of the absolute refractory period. Late in the relative refractory period, in the area of the major dip obtained by bipolar stimulation, there occurred a slight dip, a flattening, or no detectable asymmetry.

*Monopolar stimulation with stigmatic anode.* These curves sometimes showed a slight dip or flattening but usually no asymmetry early in the relative refractory period in the area of the primary or high-threshold dip. Frequently, however, the early phases of the relative refractory period could not be tested by the less effective 'anodal' stimulation. Late in the relative refractory period the stigmatic anode revealed a very definite dip in the major dip area. The absolute refractoriness of the heart to this form of stimulation was much longer and after full recovery of excitability thresholds were appreciably higher. In the area of the major dip, however, thresholds as measured by a stigmatic anode are definitely lower than thresholds to stigmatic cathodal stimulation.

There is thus a change in relative susceptibility to stimulation with the 'anodal' and 'cathodal' stigmatic electrode. Early in the cycle stimulation by a stigmatic cathode is commonly more effective. In the terminal phases of the relative refractory period and after full recovery this form of stimulation is invariably more effective; but in the major dip area the heart is more susceptible to stimuli when from a stigmatic anode.

**Bipolar stimulation.** When both anode and cathode are on the heart the thresholds to stimulation correspond to the most effective portions of the cathodal and anodal curves. The curves obtained by bipolar stimulation can be considered composite curves. Evidence indicates that in the area of the major dip the propagated impulse originates from the anodal surround. Earlier in the cycle the impulse most frequently originates from the region of the cathode. Following the major dip, during the terminal phases of the relative refractory period, and during diastole there is always a cathodal site of impulse origin. There thus appears to be a shift in sensitivity to the peculiarities of current flow maintaining in the cathodal and anodal surround. Rosenbluth (12) and others have described true primary anodal stimula-

Fig. 3. RELATIONSHIP of electrogram forms to periods of excitability in *experiment 105*. Inset is diagram of ventricles showing position of testing electrodes ( $T-$ , cathode, and  $T+$ , anode) and pick up leads. 1. Configuration of electrogram when impulse originates in cathodal surround. 2. Configuration impulse originates in anodal surround. In all three tracings, stimulus artifact includes initial diphasic wave and sloping plateau. Complex 1 is of the QS type, complex 2 of the QRS type. The  $T$  in 1 is far greater in amplitude than in 2.



tion of tissue. In our experiments we can merely state that the site of beat origin shifts from cathode to anode at a certain period of the cycle.

The evidence supporting these conclusions is as follows: Leads placed strategically to indicate origin of propagated activity in the region of cathode and anode show that the impulses do originate as indicated above. The shift from a cathodal to anodal site and back again is revealed by the change in configuration of the electrogram recorded by leads placed as indicated in figure 3. Even when monopolar stimulation is used there is some shift in site of impulse origin as shown by Harris and Moe (13). These shifts should be thought of as being caused by the changing state of the heart muscle rather than as the cause of the change in excitability observed at that time.

In experiments in which the standard arrangement of electrodes produced definite dips certain rearrangements of electrodes created conditions unfavorable for their demonstration. But even under these unfavorable circumstances some

flattening of the curves often appeared in the dip areas. It is felt that this demonstration of possible unfavorable electrode placements does not vitiate the evidence that asymmetries of excitability do characteristically occur during the recovery phases of the ventricular excitability cycle. This type of work is being continued in an effort to obtain explanations of these phenomena.

In conclusion it can be said that there are not only oscillations or asymmetries in the recovery of excitability but also that there are changes in sensitivity to different forms of stimulation (anodal versus cathodal stigmatic electrodes).

4. *Latency.* The dip area is really a period of the cycle in which a long persisting local excitatory process can be built up with greater ease than at a temporally adjacent later interval. The propagated response to a testing stimulus is not immedi-

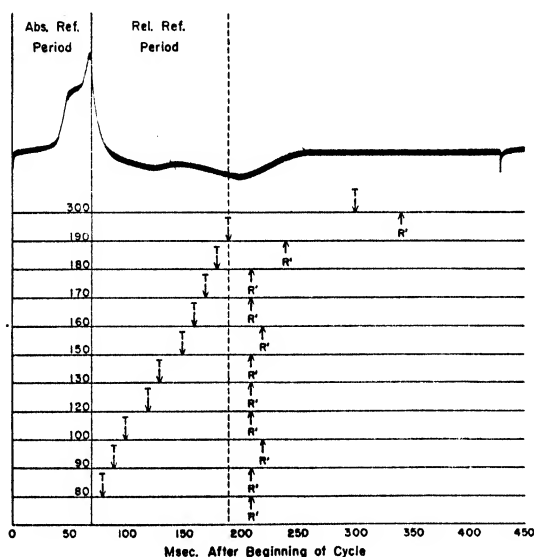


Fig. 4. INCREASING LATENCIES created by advancement of stimulation earlier into cycle. Upper tracing is EKG with R wave and inverted T. Arrows marked by T indicate instant of applying test shock; those marked by R', instant of response. Figures on horizontal lines correspond to instant of test-shock application as measured in msec. after driving stimulus.

ate but appears only after a latency which grows progressively longer as stimuli are placed earlier and earlier in the refractory portion of the cycle. Responses tend to occur at about the same time in the cycle and cannot be advanced earlier than the end of the relative refractory period (fig. 4). It appears that a stimulus to be effective must create a local excitatory state which persists until the heart attains its physiologically normal threshold. Long-duration stimuli are more effective because they can produce longer lasting excitatory processes. In none of our experiments on latency did the evidence from limb leads or from auricular and ventricular electrograms suggest supra-ventricular origin of the beat.

The terms 'absolute' and 'relative refractory period' have been criticized on numerous occasions (14, 15). Use of the expressions 'irresponsive period' (14) and 'effective refractory period' (16-18) has been proposed. Work such as described here makes it apparent that the boundaries of the so-called absolute and relative re-

fractory periods are variously defined by rectangular stimuli of different durations. However, all durations of stimulus shock reveal similar total refractory and irresponsive periods. The boundary line between normal excitability and reduced excitability is rather uniformly defined despite the fact that each stimulus duration gives its own absolute-relative refractory period border. When stimuli of 0.1 msec. duration were employed the absolute refractory period was long and the relative refractory period short. Stimulus shocks 13.0 msec. in duration gave a very brief or no absolute refractoriness and a long relatively refractory period. This evidence suggests that use of longer-duration stimuli and a stimulator of unlimited capacity (our stimulator delivered a maximum of 30 ma.) would show no absolute refractoriness to exist in any case. Even with the limited output of our stimulator we found no absolute refractoriness in 3 dogs and frequently absolute refractoriness of only 10 to 20 msec. duration in other experiments.

The local excitatory response to long-duration high-intensity stimuli can outlive the refractory period of tissues (15, 19) and either produce multiple firing (12, 20, 21)

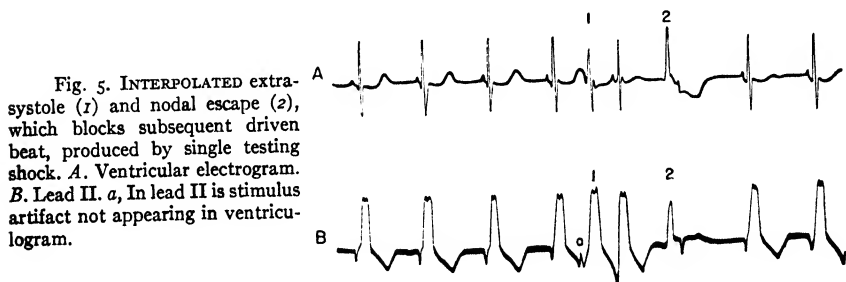


Fig. 5. INTERPOLATED extrasystole (1) and nodal escape (2), which blocks subsequent driven beat, produced by single testing shock. A. Ventricular electrogram. B. Lead II. a, In lead II is stimulus artifact not appearing in ventriculogram.

or, in the case of the heart, summate with a local intrinsic excitatory phenomenon to produce impulse origin as indicated by nodal escape in some of our experiments (fig. 5). Wégria, Moe, and Wiggers (22) found, as did we in testing cardiac excitability, that testing shocks appear to have some carry-over into subsequent cycles; therefore, to obtain uniformity of thresholds the test shocks were applied only every 7th to 11th cycle.

These observations certainly indicate that there are intervals of the cycle during which the heart cannot respond to excitatory processes and strongly suggest that there are no intervals during which excitatory processes cannot be produced in heart muscle. There is an irresponsive period and a relatively refractory period but no absolutely refractory period in terms of the ability of stimuli to create a local excitatory process which may eventually become effective.

5. *Supernormality.* A period of supernormality following the relative refractory period has been described by numerous individuals (2-5). It is best seen in certain conditions and not at all in others (17, 23). Since this supernormality has been thought to coincide with the terminal portion of the ventricular complex (4), Hoff has suggested that the state may be created by a negative afterpotential (6).

In our experiments very little evidence of supernormality of the ventricle was found. In a total of 52 experiments performed to date changes which conceivably



might be called supernormality were rare. In no case was there a pronounced lowering of the threshold. Throughout this large series of experiments there were instances of anoxia and other abnormalities which conceivably might have created supernormality but did not.

The occurrence of oscillations in the excitability threshold during recovery phases of the cycle suggests that supernormality might be merely a continuation of these fluctuations into the early phases of the post-refractory period.

#### DISCUSSION

Analysis of the changes causing the dip phenomenon presents difficulties because of the complexity of the tissue involved. The phenomenon may actually result from the heterogeneous admixture of excitable tissues and/or possible changes in distribution of current flow from testing stimuli during the cycle. In terms of the technique used, however, there is an oscillation of excitability during the relative refractory period. It is not unreasonable to assume that oscillations do occur in the processes involved in reestablishment of membrane polarization and normal excitability. Spontaneous oscillations in potential occur in skeletal muscle and nerve under certain imposed conditions (24, 25); slow phasic swings of electrical potential have been detected in smooth muscle (26) and in isolated mammalian auricular and ventricular muscle (13, 27, 28). These oscillations consist of both prepotentials associated with intrinsic excitatory processes and afterpotentials. According to Bozler's figures (26) these oscillations in potential which occur following a propagated impulse persist for 3 to 5 seconds, have a cycle of 200 msec. duration and a gradually diminishing amplitude. Bozler (28) states that oscillations in tonus and electrical potentials are characteristic properties of cardiac muscle and he explains them on the basis of fluctuations in metabolic processes. The period of the oscillations which our methods have recorded and as expressed by the intervals between dips during the relative refractory period is in the order of 30 to 80 msec. This rate appears to be intermediate between the oscillations reported by Bozler and those recorded by Harris and Moe (13) adjacent to stimulating electrodes following direct current flow. It is our concept that the asymmetries we have detected in the recovery curves represent inconstancy or incomplete dominance of the repolarization processes.

Since any stimulus applied during the relative refractory period can only act after a long latency it must act by establishment of a long-lasting excitatory state. The nature of the dip and the causes of the asymmetries of recovery process cannot be explained until more information can be obtained concerning the nature of excitation and the factors involved in the latency phenomenon. Studies of latency have been undertaken on numerous occasions (14, 15, 17, 19). Latency conceivably can be due to a complex of factors such as time involved in the building up of the excitatory process, local spread of an excitatory potential and conduction of the impulse to pick up electrodes. Moe, Harris and Wiggers (19) state that tissue polarization resulting from strong brief shocks applied during refractoriness may persist until early diastole and produce the latent response. Stronger longer-lasting stimuli should produce a greater degree of polarization. Since the most effective stimuli early in the cycle produce effects from the cathodal surround, it is suggested that the per-

sistent excitatory process which eventually produces the latent response is of the nature of a persistent local depolarization or sink. As recovery occurs in the surround it is enabled to give origin to late or even multiple firing.

There is no question that stimuli can produce excitatory states during the irresponsive period even though not evoking a response. An ineffective local state thus created will summate with the excitatory effect of a second sub-threshold stimulus to produce an eventual response (29). Whether the dip is diagnostic of conditions permitting easier establishment or easier development and spread of the local excitatory response cannot as yet be stated. It is not known whether the peculiarities of the major dip are shared by the primary and rare secondary deflections. They may originate from different causative factors but in terms of testing stimuli they all represent recovery of a degree of excitability which cannot be maintained and all are followed by a period of reduced irritability before recovery processes again dominate and progressively restore excitability to the normal value. In speaking of the responses to stimuli placed early in the cycle Lewis (17) stated that such a stimulus may yield a response while another placed only slightly later in the cycle may not. This indicates that he too observed some asymmetry of the recovery process.

#### SUMMARY

New methods of testing the excitability of the mammalian ventricle throughout the cardiac cycle reveal asymmetrical recovery of excitability during the relative refractory period. It is characteristic of the recovery process that the muscle periodically attains a degree of excitability which is not sustained. Periods of reduced excitability precede further recovery. Although there are sometimes two and rarely three deflections or dips, usually only one major dip is present. The nature of this dip phenomenon and the processes involved in the production of responses to stimuli applied during the refractory period are discussed.

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# FORMATION OF BRADYKININ IN ANAPHYLACTIC AND PEPTONE SHOCK

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**R**OCHA E SILVA, Beraldo and Rosenfeld (1) have reported the formation of a smooth muscle stimulating and hypotensive factor from the globulin fraction of the blood by the action of certain snake venoms and by trypsin. This factor (bradykinin), which is similar to but probably not identical with the slow-reacting substance (SRS) of Feldberg and Kellaway (2, 3), is characterized as follows: *a*) it produces a slow type of contraction of guinea pig, rat and rabbit intestine and uterus which is not antagonized by either antihistaminics or atropine; *b*) it causes a fall in blood pressure in cats, rabbits and dogs; *c*) it is dialyzable, stable to heat in neutral or slightly acid solution but not in concentrated hydrochloric acid, is insoluble in ether, acetone and is destroyed by incubation with venom or trypsin; and *d*) the guinea pig intestine shows no desensitization on repeated additions of bradykinin to the perfusion bath.

In view of the similarity of many of the phenomena of anaphylactic and peptone shock in dogs to the reactions produced by venom and trypsin, it was decided to ascertain whether bradykinin is found in significant amounts in these reactions.

## EXPERIMENTAL

*Anaphylactic Shock.* Eighteen dogs were sensitized to either horse serum or egg albumin. The oxalated blood samples collected before and several minutes after the shock were tested for smooth muscle activity on the guinea pig intestine immersed in 7 cc. Tyrode solution in a smooth muscle bath. In order to inhibit the activity due to histamine, 2 or 3 micrograms of Benadryl were added to the bath and after one minute the muscle was washed with Tyrode solution. Before and after the addition of the blood samples to the bath, the muscle was tested with a histamine standard solution. In addition to the test with an antihistaminic, an alcoholic extract of the blood samples was incubated with trypsin<sup>2</sup> or snake venom<sup>3</sup> in order to characterize the active principle. This extract was prepared by extraction of active plasma (10 cc.) with 2 volumes of boiling alcohol. The mixture was boiled 10 minutes and filtered. The filtrate was concentrated *in vacuo* and dried. The dried extract was re-extracted with distilled water and the final volume restored

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<sup>2</sup> The crystalline trypsin used was an Armour preparation containing 50 per cent of magnesium sulfate.

<sup>3</sup> Supplied by Butantan Institute, S. Paulo, Brazil.

to the original plasma volume. This extract was incubated with trypsin or venom in proportion of 500  $\mu\text{g}/\text{cc.}$  for 6 to 8 hours at 37°C.

For the purpose of comparing the activity of blood samples, a bradykinin standard<sup>4</sup> has been used and the assay upon the guinea pig ileum has been most useful. The response of the intestine is fairly quantitative allowing satisfactory comparison between the unknown and the standard.

Bradykinin activity was present in the blood of 7 out of 18 dogs after the injection of antigen, the amount of bradykinin ranging between 20 to 90  $\mu\text{g.}$  of bradykinin standard per cc. of blood. The amount of bradykinin found in the blood bears no definite relationship to either the amount of histamine released or to the severity of the shock. In some experiments no gut stimulating effect could be detected in the blood samples tested immediately after they were withdrawn from the dog. However, the activity could be observed after 10 to 30 minutes incubation of the blood samples at 37°C.

*Peptone Shock.* Twenty dogs were injected intravenously with 10 per cent Bactoprotone solution, in doses of 0.5 to 5 cc/kg. The solution was boiled for 10 minutes, cooled and shaken with permutit in order to remove traces of histamine. Bradykinin activity was present in the blood of 17 out of 20 dogs after peptone shock, ranging between 30 to 120  $\mu\text{g.}$  of bradykinin standard per cc. of blood. In peptone shock as in anaphylaxis the bradykinin activity bears no relationship to the amount of histamine released or to the severity of the shock. Figure 1 shows a blood pressure tracing during peptone shock and contractions of guinea pig ileum after additions of blood samples to the bath before and at several intervals after the injection of peptone. The maximum activity is shown to be attained at 90 minutes. The blood samples withdrawn later show progressively decreasing activity which finally disappears.

A powder obtained by alcoholic extraction of plasma drawn from a dog during peptone shock produces a fall in blood pressure in dogs and rabbits. In one experiment only traces of active substance could be detected in the blood after 0.5 cc/kg. of peptone. After recovery from the first shock a second injection of 1.5 cc/kg. produced activity equivalent to 30  $\mu\text{g.}$  of bradykinin/cc. of blood. The blood pressure fell 50 mm. Hg. This experiment can be understood on the basis of a direct action of peptone on the globulin in the animal body, eliciting the formation of bradykinin by a mechanism apparently independent of the liberation of stored substances such as histamine and heparin, which may be depleted by the first shock so that little may be left for subsequent reactions. Furthermore, the fact that a larger dose of peptone produces larger amounts of active substance when injected in the same animal suggests that the blood activity can be related to the amount of peptone injected, another fact supporting the hypothesis of direct action of peptone on the globulin.

*In vitro formation of gut stimulating substance by addition of peptone to globulin.* The addition of peptone solution to oxalated dog plasma led to the formation of a

<sup>4</sup> The bradykinin standard employed was a six-month-old crude preparation (1) which was still active upon the isolated gut of guinea pig and caused a fall in the blood pressure of rabbits and dogs.

muscle stimulating agent like that detected in the blood of dogs after the injection of peptone. The plasma globulins, precipitated by half saturation with ammonium sulfate were shown to be responsible for the plasma activity. The cells and albumin fraction were found to be inactive when incubated with peptone. When added to the bath, 0.02 to 0.04 cc. of fresh 10-per cent solution of Bactoprotone pretreated with permutit, commonly produced no response at all or very moderate responses of the guinea pig intestine which became desensitized after the second or third addition of peptone. The globulin solution could be added to the bath without eliciting any

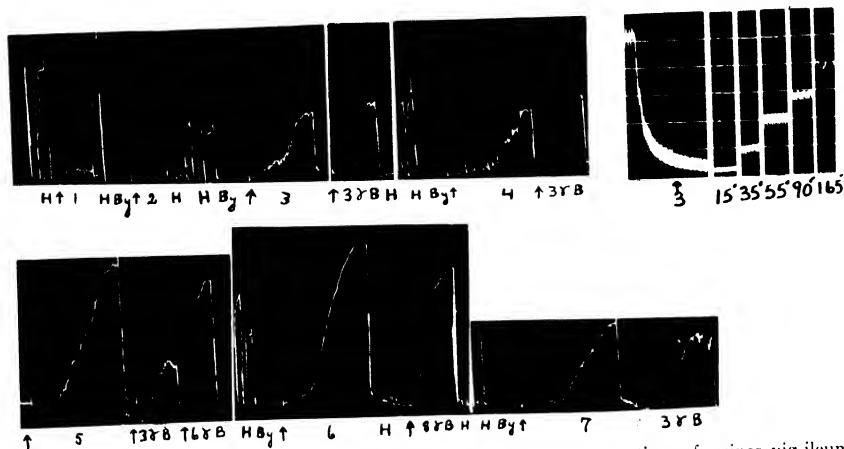


Fig. 1. BLOOD PRESSURE TRACING during peptone shock and contractions of guinea pig ileum. 1 = 0.3 cc. blood before injection of peptone. 2 = 0.3 cc. blood 3 min. after shock. 3 = 0.3 cc. blood 15 min. after shock. 4 = 0.3 cc. blood 35 min. after shock. 5 = 0.3 cc. blood 55 min. after shock. 6 = 0.3 cc. blood 90 min. after shock. 7 = 0.3 cc. blood 165 min. after shock. H = 0.2 cc. histamine 1:2 million. By, 2  $\mu$ g. Benadryl. B, bradykinin.

response. However, when 0.2 cc. of peptone solution was added to 2 cc. of globulin and incubated for 1 to 2 minutes, a contraction of guinea pig ileum could be observed after adding 0.2 cc. of the mixture to the bath. The activity increased progressively, reaching a maximum after 2 to 3 hours of incubation at 37°C. (fig. 2).

A decrease in activity was sometimes observed when the incubation period was prolonged for 5 to 6 hours, but activity rarely disappeared completely. The optimum amount of peptone was found to be between 0.1 to 0.2 cc. of a 10-per cent solution for each cc. of globulin solution. The globulin solution was made up to about  $\frac{2}{3}$  of the original plasma volume. If a larger dose of peptone was used, no further increase in activity could be observed. A 10-per cent solution of Bactoprotone (Difco) was only half as active as a 10-per cent Bactoprotone (Difco) solution.

*Influence of pH and temperature upon the formation of the active substance, in vitro.* Three samples of globulin solution were adjusted with 1N HCl and NaOH to pH 8.4, 7.4 and 6.4, respectively. Since the globulin acts as a buffer, very small change in pH took place when the peptone solution was added. The pH of the mix-

ture was readjusted and the samples were incubated at  $37^{\circ}$ . The rate of formation of active substance was determined on the guinea pig ileum by comparison with a bradykinin standard. Control experiments showed that the buffer capacity of Tyrode solution prevented any change in pH on addition of 0.2 cc. samples to the bath. As shown in figure 3, *I*, the optimum pH for the formation of the active substance was found to be 7.4 which suggests the optimum pH to lie between 6.4 and 8.4. In

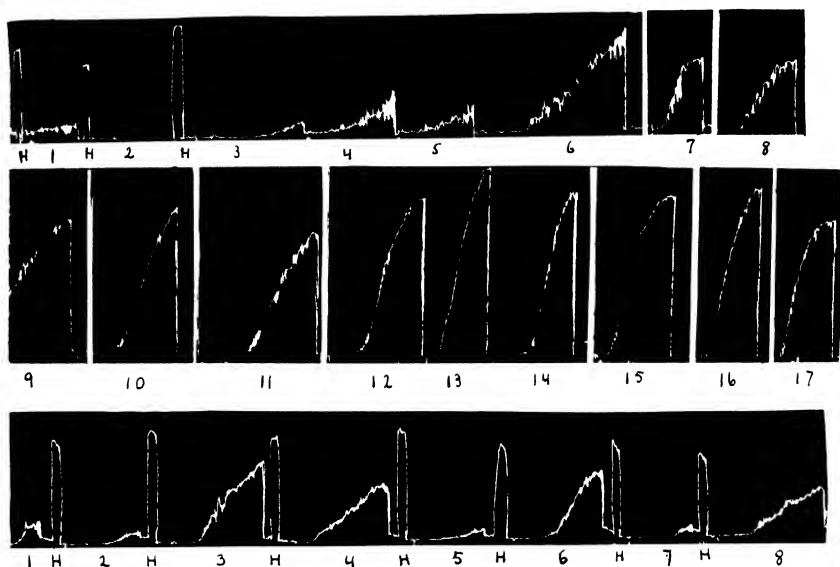


Fig. 2. *Upper and middle panel:* contractions of guinea pig ileum. 1 = 0.02 cc. 10% peptone solution; 2 = 0.3 cc. globulin solution; 3, 4 = 0.2 cc. of a mixture (2 cc. globulin + 0.2 cc. peptone solution) after 1 and 12 minutes of incubation; 5 = 25  $\mu$ g. bradykinin standard; 6, 8, 9, 11 = 0.2 cc. of mixture after 60, 80, 105 and 110 minutes of incubation; 7, 10 = 60 and 100  $\mu$ g. bradykinin standard; 12, 14, 15, 17 = 0.2 cc. of same mixture after 210, 220, 440, 450 minutes of incubation; 13, 16 = 120 and 150  $\mu$ g. bradykinin standard. *Lower panel:* contractions of guinea pig ileum. 1 = 0.2 cc. globulin solution; 2 = 0.04 cc. 10% peptone solutions; 3, 4, 6, 8 = 0.2 cc. of a mixture (2 cc. globulin + 0.4 cc. peptone solution) after 3, 5, 10 and 15 minutes of incubation. 5, 7 = 0.2 cc. of a mixture (2 cc. globulin + 2 mg. soybean trypsin inhibitor + 0.4 cc. peptone) after 5 and 10 minutes of incubation. H = 0.2 cc. histamine 1:2 million.

addition to pH, the temperature has an important influence on the formation of the active substance, as shown by figure 3, *II*. For a 7-minute incubation period the optimum temperature was found to lie between  $40^{\circ}$  to  $50^{\circ}\text{C}$ .

*Inhibitory effect of soybean trypsin inhibitor.* Crystalline soybean trypsin inhibitor<sup>5</sup> when added to a globulin solution in the proportion of 1 to 2 mg./cc. completely inhibited the appearance of gut stimulating substance after addition of peptone (fig. 2, *lower tracing*). Control experiments showed that soybean trypsin inhibitor in the amounts used in this experiment has no effect upon the guinea pig ileum itself nor upon the effect of the bradykinin standard.

<sup>5</sup> Kindly supplied by Dr. F. C. McIntire, Abbott Laboratories, North Chicago, Ill.

Heparin showed a very weak inhibition in amounts as high as 10 to 20 mg/cc. of globulin solution.

*Formation of bradykinin by fibrinolysin*<sup>6</sup>. Addition of small amounts of fibrinolysin (1-2 mg.) to the bath commonly elicited no contraction at all or very moderate responses of the guinea pig gut which became desensitized after a second or third addition of fibrinolysin. If 4 to 5 mg. of fibrinolysin were added to 2 cc. of globulin, a contraction could be observed after 1 to 2 minutes of incubation. If the incubation was prolonged for 2 to 3 hours, the active principle disappeared, thus indicating that it had been destroyed by the prolonged action of the fibrinolysin (fig. 4, I). Soybean trypsin inhibitor blocks the formation of gut stimulating substance from

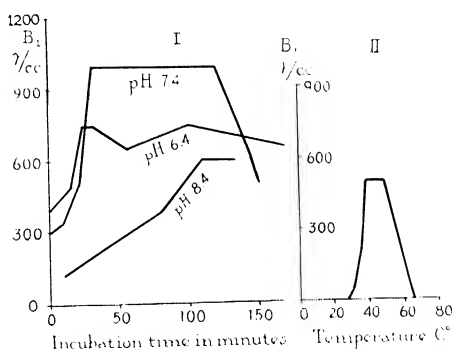


Fig. 3. INFLUENCE OF pH AND TEMPERATURE on formation of gut stimulating substance by incubation of globulin and peptone. Ordinates: amounts of active substance/cc. of peptone globulin mixture expressed as bradykinin standard B<sub>1</sub>; abscissas: I) incubation time in minutes; II) temperature. Incubation time in experiment II, 7 minutes. Temperature in experiment I, 37°.

globulin by fibrinolysin similarly to its inhibition of the action of peptone on globulin (fig. 4, II).

#### DISCUSSION

The evidence presented indicates that in addition to histamine another hypotensive and smooth muscle stimulating principle is formed during the course of anaphylactic and peptone shock in dogs and when plasma globulin is incubated with peptone, *in vitro*. The results obtained so far permit the conclusion that this substance has many of the characteristics of bradykinin, with which it is probably identical.

The first question raised is whether this substance plays some important role in the mechanism of anaphylactic and peptone shock or whether its formation is a consequence of, rather than a causal factor, in shock. For one of the anaphylactic shock experiments the blood sample withdrawn 2 minutes after the injection of antigen and immediately tested on guinea pig intestine showed great activity (50 µg/cc. compared with bradykinin standard). The total amount of active substance

<sup>6</sup> Kindly supplied by Dr. G. Ungar, Rheumatic Fever Research Institute, Northwestern University, Chicago, Ill. and by Eugene C. Loomis, Parke, Davis & Co., Detroit, Mich.



calculated to be present in the circulating blood of this dog was estimated to be equivalent to 4 mg/kg. of bradykinin standard. Such an amount of bradykinin standard produces an appreciable fall in blood pressure, when injected in dogs and rabbits. On the other hand, figure 1 shows an experiment in which the blood sample withdrawn 90 minutes after the shock showed maximum activity, which, of course, does not coincide with the maximal fall in blood pressure. Furthermore, the blood pressure tended to recover although the amounts of active substance increased

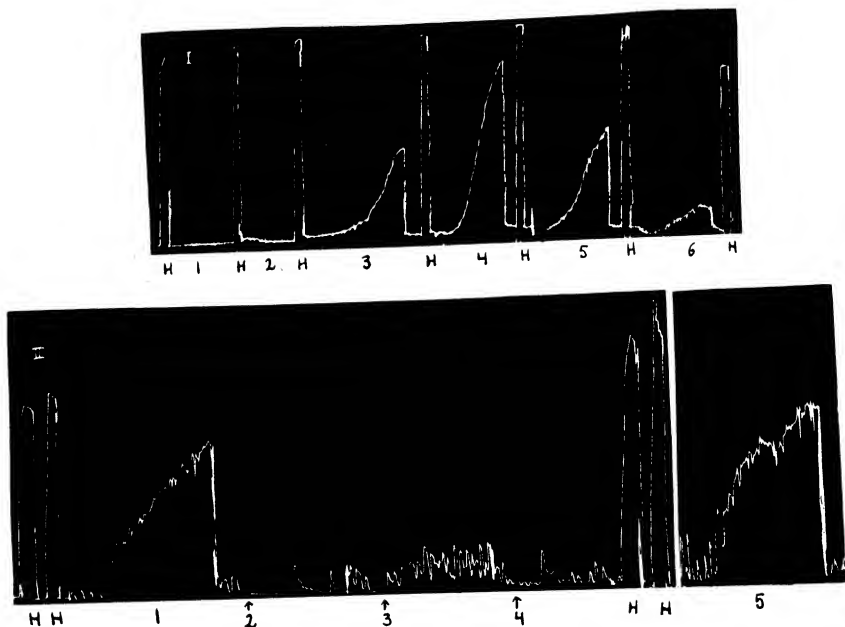


Fig. 4. CONTRACTIONS OF GUINEA PIG ILEUM. I) 1 = 0.2 cc. globulin; 2 = 2 mg. fibrinolysin; 3 to 6 = 0.2 cc. of a mixture (10 cc. globulin + 60 mg. fibrinolysin) after 12, 30, 120 and 225 minutes of incubation. II) 1, 5 = 0.4 cc. of a mixture (3 cc. globulin + 16 mg. fibrinolysin) after 2 and 8 minutes of incubation; 2 to 4 = 0.4 cc. of a mixture (16 mg. fibrinolysin + 10 mg. soybean trypsin inhibitor + 3 cc. globulin) after 2, 7 and 8 minutes of incubation.  
H = 0.10 cc. histamine 1:2 million.

in the blood. This fact is in contrast with our previous experience in which we obtained significant blood pressure drops in dogs injected with amounts of bradykinin released in the experiment shown in figure 1 at 90 minutes after shock. Since it is a well known fact that epinephrine is released after the injection of histamine (4-6), it may be possible that some hypertensive substance or substances released during the shock can counteract the effect of bradykinin.

The bradykinin-like substance found in the circulating blood of dogs during anaphylactic and peptone shock does not appear to be a very important factor in the overall mechanism of such shocks. However, it may constitute an aggravating factor and may be concerned with some of the phenomena of anaphylaxis.

Schild has shown (7) that the addition of antigen to the uterus of a sensitized guinea pig suspended in a perfusion bath leads to liberation of histamine from the uterus, but that the uterine contraction so produced is not wholly accounted for by the histamine release.

Wells *et al.* (8), in studying the effect of Benadryl on peptone shock in dogs and guinea pigs, concluded that this drug does not prevent completely the vascular shock produced by the injection of peptone. The same authors (9), studying the effect of Benadryl against anaphylaxis in dogs, did not exclude the possibility that factors other than histamine may be concerned in anaphylactic shock in dogs.

Another question which arises concerns the mechanism of the formation of the active principle in anaphylactic and peptone shock and *in vitro*, when peptone is added to plasma globulin. It has been shown that activation of fibrinolysin occurs during anaphylactic and peptone shock in dogs (10) and *in vitro* when guinea pig serum is put into contact with peptone or antigen (11).

We were able to demonstrate the *in vitro* formation of bradykinin when fibrinolysin was added to plasma globulin. Soybean trypsin inhibitor inhibits the formation of bradykinin which occurs normally on addition of peptone to globulin or by the action of fibrinolysin on globulin. These experiments suggest that fibrinolysin, activated during anaphylactic or peptone shock or *in vitro*, is the enzyme responsible for the formation of bradykinin or bradykinin-like substance appearing in these reactions.

#### SUMMARY

A hypotensive and smooth muscle stimulating substance which presents pharmacological properties similar to bradykinin has been found in anaphylactic and peptone shock in dogs and *in vitro*, when peptone is added to plasma globulin. Fibrinolysin activated during these reactions is assumed to be the enzyme responsible for the formation of bradykinin-like substance. The possible role of bradykinin in anaphylactic and peptone shock is discussed.

It is a pleasure to acknowledge the interest and helpful suggestions of Dr. Carl A. Dragstedt, Dr. Karl F. Urbach and Dr. J. A. Wells. I am indebted to Mrs. I. O. Beraldo for her technical assistance.

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# PRESSOR PATHWAYS NOT BLOCKED BY TETRAETHYLAMMONIUM<sup>1</sup>

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IN A recent study it was shown that administration of tetraethylammonium (TEA) fails to block completely the cardiac chronotropic and inotropic effects of preganglionic sympathetic stimulation in the dog (1). The conclusion was drawn that sympathetic impulses not blocked by TEA could traverse a qualitatively different type of ganglionic synapse, or course directly to the heart without synaptic interruption. In the present study similar pathways have been demonstrated in the vasomotor system of the dog and rabbit, but not in the rhesus monkey and cat.

## METHODS

Experiments were made on 12 dogs, 1 cat, 3 rabbits, and 3 monkeys. Under thiopental-barbital or pentobarbital anesthesia, the spinal cord was exposed and transected at various levels in the thoracic region. Artificial respiration was maintained by means of a Starling pump, and the animals were completely curarized by the administration of repeated doses of D-tubocurarine. Arterial pressure was recorded on a smoked drum with a mercury manometer. Electrodes were inserted into the cord caudal to the point of section, and maximal stimuli at a frequency of 15/second were applied at intervals from an Electrodyne stimulator.

## RESULTS<sup>4</sup>

*Dog.* Stimulation of the cord in the lumbar region caused a moderate rise of arterial pressure. Following administration of TEA in repeated doses beginning with 5 mg/kg., the pressor response to cord stimulation was slightly reduced but never abolished. Stimulation at higher levels caused a greater pressor response accompanied by an increase of heart rate. TEA only slightly reduced the pressor response but greatly reduced the heart-rate response to stimulation (fig. 1). The administration of pentamethonium iodide (C-5) likewise failed to prevent the pressure rise, but the adrenolytic agent  $\alpha$ -naphthylmethylethyl- $\beta$ -bromoethylamine hydrobromide (SY-28) completely blocked the response (fig. 1).

In five experiments the cord was exposed from mid-thoracic to low lumbar levels,

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<sup>2</sup> Adapted from a thesis submitted by W. A. F. to the faculty of the Horace Rackham School of Graduate Studies of the University of Michigan in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>3</sup> Present address: Department of Physiology, New York State University College of Medicine at Syracuse.

<sup>4</sup> Tetraethylammonium chloride, pentamethonium bromide, and SY-28 were generously supplied by the Research Laboratories, Parke Davis and Company, Detroit.

crushed and ligated at the caudal end, and transected at the cephalic end of the exposed area. After recording pressor responses to stimulation before and after administration of TEA, all dorsal roots attached to the isolated length of cord were cut. This procedure caused no significant reduction of the pressor response (fig. 2).

In three experiments prepared as above, the ventral roots were cut, leaving the dorsal root pathways intact. After this procedure, cord stimulation caused no elevation of arterial pressure (fig. 3).

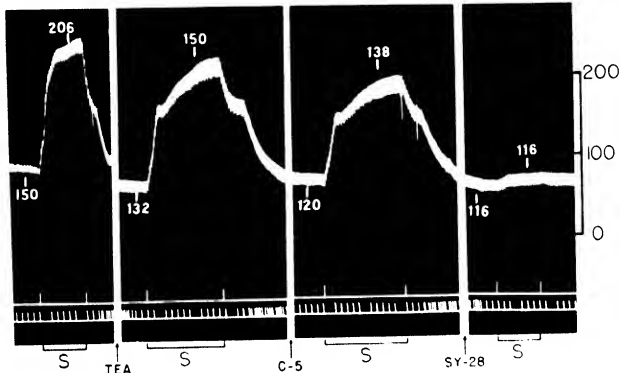
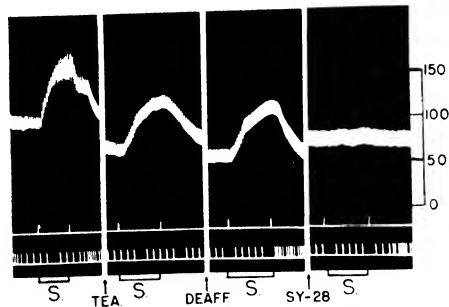


Fig. 1. *Experiment 1/31/50.* Dog, 6.8 kg., thiopental-barbital anesthesia, curarized, vagi cut, cord sectioned at D-5. *Tracings:* carotid arterial pressure, signal and time in 10-second intervals. Figures refer to heart rate per minute. At S, stimulation of cord at D-6. *TEA*, C-5, and SY-28 indicate injections of tetraethylammonium chloride, 15 mg/kg.; pentamethonium bromide, 1.5 mg/kg.; and SY-28, 1 mg/kg.

Fig. 2. *Experiment 2/15/50.* Dog, 13 kg., thiopental-barbital anesthesia, curarized, cord sectioned at D-7 and L-5. *Tracings:* as in figure 1. At S, stimulation at D-8. *TEA*: tetraethylammonium chloride, 12 mg/kg.; *DEAFF*: dorsal roots cut from D 7 to L-5; *SY-28*: 1.5 mg/kg.



*Rabbit.* Similar results were obtained in three experiments on rabbits. The pressor response to cord stimulation was never abolished by TEA, but was eliminated by SY-28 (fig. 4.4). No attempt was made to determine whether the pressor pathways leave the cord by way of the ventral roots in this species.

*Cat.* Since it had already been demonstrated that TEA blocks the pressor effect of cord stimulation in the cat (2), only one experiment was performed on this species in the present series. In confirmation of the previous work, it was found that the pressor response was completely blocked by TEA.

*Monkey.* Three monkeys were prepared in a similar manner. The response to cord stimulation was easily prevented by moderate doses of TEA (fig. 4B).

#### DISCUSSION

Though cardiovascular reflexes initiated from the carotid and aortic pressure-sensitive areas are blocked by TEA in the dog (3), it is apparent that an auxiliary pressor system invulnerable to this agent exists in this species and in the rabbit. These observations extend the previous demonstration of cardiac sympathetic pathways which resist interruption by ganglionic blocking agents in the dog.

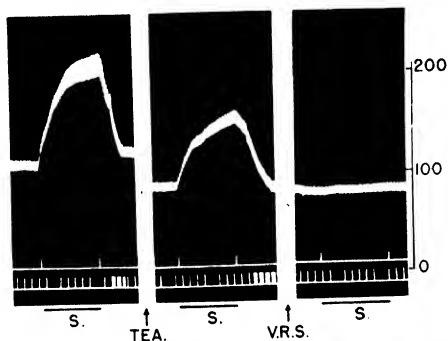
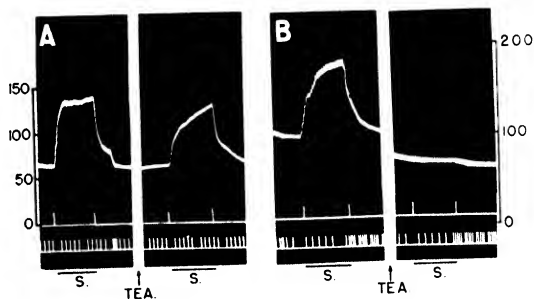


Fig. 3. Experiment 5/25/50. Dog, 15 kg., thiopental-barbital anesthesia, curarized, cord sectioned at D-3 and L-5. Tracings: as in figure 1. At S, stimulation at D-4. TEA: tetraethylammonium chloride, 10 mg/kg.; V.R.S.: ventral roots sectioned from D-3 to L-5; dorsal roots intact.

Fig. 4. A. Experiment 6/19/50. Rabbit, 4.3 kg., pentobarbital anesthesia, curarized, cord sectioned at D-1. Tracings: as before. At S, stimulation at D-2. At T: TEA, 17 mg/kg. B. Experiment 2/27/50. Monkey, 3.1 kg., pentobarbital anesthesia, curarized, cord sectioned at D-3. Tracings and signals: as before. At TEA: tetraethylammonium 8 mg/kg.



The auxiliary pressor path has been shown to leave the cord by way of the ventral roots, but its further distribution has not yet been delineated. In the case of the cardiac pathways, the un-blocked fibers course through the thoracic sympathetics, which indeed would be the only available channels, but it is by no means certain that the vasoconstrictor pathways do not pass directly to the spinal nerves without entering the ganglionated chains. Such a direct course could well explain the persistence of vasoconstrictor reflexes in 'sympathectomized' extremities in the dog, as recently described by Randall *et al.* (4).

The possible existence of similar mechanisms in the human subject could explain the observation that TEA never causes a peripheral vasodilatation comparable in magnitude with that resulting from paravertebral block with local anesthetics (5), and might explain the failure of TEA to offer a satisfactory prediction of the depressor response to extensive sympathectomy in hypertension (6).

Failure of TEA to block the pressor response to asphyxia in the dog (7) is probably due to the intervention of the auxiliary vasomotor mechanisms, a possibility which is the subject of a separate communication (8).

#### SUMMARY

Stimulation of the spinal cord provokes an increase of arterial pressure which can be blocked by tetraethylammonium in the cat and monkey but not in the dog and rabbit. In the dog the pressor pathway leaves the cord through the ventral roots.

The authors express their appreciation to Mr. Samuel Irwin for his assistance in some of these experiments.

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# PREGNANCY TEST UTILIZING FROGS AND TOADS

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TAIPÉH, FORMOSA

THE reports of Mainini (1), utilizing the male South African toad (*Bufo arenarum* Hensel), and Wiltberger and Miller (2), utilizing male frogs (*Rana pipiens*), in the diagnosis of pregnancy led the author to investigate the use of the common frogs and toads of Formosa in the diagnosis of pregnancy and sex-hormone producing tumors.

## METHOD AND RESULTS

The number and species of experimental animals consisted of the following: 214 *Rana tigerina*; 58 *R. narina*; 8 *R. plancyi*; 57 *Bufo melanostictus*; and 2 *B. bankorensis*. The animals were kept in water prior to their utilization. The urine to be tested was injected into the dorsal or lateral lymph sac, the latter being the most convenient in the toad. The injected animal was placed in a conical filter suspended in a glass column and covered by a bell jar. The animals' urine was collected in a cup beneath the filter. It was examined, without staining, under 400 $\times$  magnification. If spermatozoa were not found, the urine was discarded and a new sample obtained by opening the anus of the animal with a pincette. Although the animal spermatozoa were readily differentiated from other substances and micro-organisms, control samples of spermatozoa were taken directly from the testis in the first experiments. Water, Ringer's solution, and the urines of males and virgins were injected into control animals.

The results obtained in the 122 cases of pregnancy, amenorrhea, and other conditions are shown in table 1. The length of time necessary for the appearance of spermatozoa in the animals' urine following the injection of the test urine varied from 70 minutes to 5 hours. In general, examinations were made at the end of 2 to 3 hours and, if negative, repeated at the end of 4 to 5 hours. In positive cases, the animal continued to excrete spermatozoa for 24 to 30 hours but never longer than 48 hours. The minimal volume of test urine required to produce a positive reaction was 0.1 to 0.3 cc.

Repeated clinical observation of all cases was not feasible. Accordingly, there is a possibility of diagnostic error in the first trimester group of pregnancies and in the cases of amenorrhea. In the 3 cases diagnosed 'death of fetus,' the positive reactions are believed to be due to survival of the placenta. In the case of the chorioepithelioma exhibiting a positive reaction, a history of previous hydatid mole was given, and on operation, a tumor was found near the portio. Utilizing the frog *R. tigerina*, the urine

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of this case appeared to be 5 to 10 times as potent in producing a positive response as urine from an individual with a normal pregnancy. This corresponds roughly with the results of examinations which were made utilizing the Friedman test. The diagnosis of hydatid mole, shown in table 1, was confirmed by operation. The urine from this case appeared to be 100 to 500 times as potent as urine from a case of normal pregnancy in producing a positive frog test. None of the control animals gave a positive reaction to any of the substance used.

TABLE 1. RESULTS OF PREGNANCY TEST

CONDITION	NO. CASES	POSITIVE TEST	NEGATIVE TEST
Pregnancy			
1st trimester.....	50	48	2 <sup>1</sup>
2nd trimester.....	21	20	1 <sup>2</sup>
3rd trimester.....	23	21	2 <sup>3</sup>
Amenorrhea.....	4		4
Amenorrhea lactationis.....	2		2
Amenorrhea after induced abortion.....	1		1
Amenorrhea disfuncio ovarii.....	1		1
Dysmenorrhea membranacea.....	1		1
Abortion.....	3		3
Missed abortion.....	1		1
Death of fetus.....	3	2	1
Endometriosis.....	1		1
Metropathia hemorrhagica.....	1		1
Cystoma.....	1		1
Myoma.....	5		5
Adnexal tumor.....	1		1
Chorioepithelioma.....	2	1	1
Hydatid mole.....	1	1	

<sup>1</sup> Sixty days following onset last menses.

<sup>2</sup> Four months following onset last menses.

<sup>3</sup> Eight months following onset last menses.

#### DISCUSSION

From the results of the tests it is inferred that the chorionic gonadotropic hormone is responsible for a positive reaction in the frog and toad. Utilizing the dried, ether-washed, alcohol precipitate of urine, a yellowish white substance was obtained which, when reconstituted with water and injected into frogs, produced a positive response. This appears to confirm the role of the chorionic gonadotropic hormone.

It appears that no special type of frog is required for the pregnancy tests and that the reliability of the test is high. The 50 per cent false negative reactions obtained by Wiltberger and Miller (2) in the last trimester of pregnancy might be accounted for by the different species of test animal used and the environmental temperature. The tests described in this paper were made in the months of May through September.

The assumption that the active factor is the chorionic gonadotropic hormone appears to be sustained by the work of Nakamura (3) who found that Prolan (Bayer) or the urine of a pregnant woman injected into a Japanese salamander in the pre-



copulation season caused the excretion of spermatozoa. Further, Robbins *et al.* (4) found that the injection of the anterior pituitary and the chorionic gonadotropic hormone into male *Xenopus laevis* caused the excretion of motile spermatozoa.

#### SUMMARY

Male *Rana tigerina*, *R. narina*, *R. plancyi*, *Bufo melanostictus* and *B. bankorensis* were used as the test animals for pregnancy test and the following results were obtained: Positive reaction of the test is 100 per cent reliable as either pregnancy, chorioepithelioma or hydatid mole, while negative reaction is minimally 94 per cent reliable; that is, in a few cases of pregnancy the test may show negative reaction. The earliest case of pregnancy which showed positive reaction was on the 34th day after the beginning of the latest menses. The test is applicable in the differential diagnosis of chorioepithelioma and hydatid mole. The principle of the test is the chorionic gonadotropic hormone.

My thanks are due to Dr. C. S. Lin from whom most of the material for the experiment was derived and also due to Mr. T. N. Li for his cooperation.

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# SURVIVAL IN THE CIRCULATION OF THE GROWTH AND ADRENOCORTICOTROPHIC HORMONES AS EVIDENCED BY PARABIOSIS<sup>1</sup>

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**L**ITTLE is known concerning the rate of formation, survival time, distribution in the body, blood levels or site of removal of many of the hormones. The interrelationships of these justify their careful investigation, for the blood level of a hormone is a function of its production rate, its distribution in the body and its rate of removal from the circulation.

Parabiosis has a peculiar advantage as a method of studying the survival of hormones in the circulation, in that naturally secreted hormones can be studied and at physiological levels. If a rat producing a hormone is united in parabiosis to a partner not producing this hormone, the concentration achieved in the circulation of the non-producing partner is a function of several factors. Careful consideration of the parabiotic relationships (1) shows that the factors influencing the distribution of a hormone between parabionts are: 1) rate of exchange of blood between the animals; 2) distribution of the hormone in the fluid spaces of the body, and 3) average life of the hormone molecule in the fluid space. A theoretical relationship including these various functions was derived for the distribution of a substance in question between members of a parabiotic pair under conditions of continuous production or administration to only one of the members. The relationships can be expressed in the word formula modified from Huff, Trautman and Van Dyke (1):

$$\frac{\text{Concentration of hormone achieved in the non-producing animal}}{\text{Concentration of hormone achieved in producing animal}} = \frac{\text{Fraction of 'hormone space' flowing through anastomosis per unit time}}{\left[ \frac{\text{Fraction of 'hormone space' flowing through anastomosis per unit time}}{\text{Fraction of 'hormone space' cleared of hormone in the non-producing animal per unit time}} \right]} + \left[ \frac{\text{Fraction of 'hormone space' cleared of hormone in the non-producing animal per unit time}}{\text{Fraction of 'hormone space' cleared of hormone in the non-producing animal per unit time}} \right]$$

or by symbols as:

$$\frac{C_H}{C_N} = \frac{a}{a + k} = \frac{\frac{a}{V_h}}{\frac{a}{V_h} + \frac{a}{V_h}} = \frac{\frac{a}{V_h}}{\frac{a}{V_h} + \frac{1}{T_r}} \quad (1)$$

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Where  $C_H$  = concentration of hormone in hypophysectomized parabiont.

$C_N$  = concentration of hormone in normal parabiont.

$a$  = cc/minute of blood flowing each way through the anastomosis.

$k$  = cc. of blood cleared of hormone/minute.

$V_h$  = 'hormone space.'

$T_T$  = turnover time or average life of the hormone molecule in the circulation.

The *first problem*, to determine accurately the rate of exchange of blood between the members, has been reported by Van Dyke, Huff and Evans (2) using  $\text{Fe}^{59}$ -tagged erythrocytes. The flow rate was found to be remarkably constant for all pairs and under varying conditions. It was found that approximately 0.66 per cent of the blood volume flows per minute from one animal to the other.

Having established the rate of exchange of blood, the *second problem* was to determine the distribution of the hormones in question in the fluid spaces of the body of a single rat. This might be called a measurement of the 'hormone space' of the body

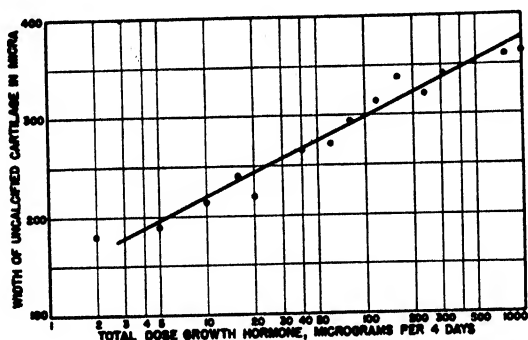


Fig. 1. EFFECT OF PITUITARY GROWTH HORMONE on width of the proximal epiphyseal cartilage of the tibia of the hypophysectomized rat.

and was judged by the extent of dilution of an intravenously administered hormone in the plasma and lymph. The *third problem* was to determine the distribution of growth hormone and adrenocorticotrophic hormone between members of parabiotic pairs. This was done either by leaving the pituitary intact in only one of the partners or by removing the pituitaries from both members and injecting the hormone into only one partner. The increase in anus-tail tip length or the resulting adrenal weights, when compared to standards, served as quantitative measurements of the amounts of hormone received by each parabiont. The function which could not be determined experimentally, the rate of removal of growth hormone and adrenocorticotrophic hormone from the circulation when they existed there in nearly physiological concentrations, remained as the only unknown in the equation. It was arrived at by substitution of the experimentally determined values, and subsequent solution for the removal rate.

#### EXPERIMENTAL ANIMALS

Female rats of the Slonaker strain which has been propagated exclusively by brother and sister inbreeding for 50 generations, were used to form the parabiotic

pairs. This degree of inbreeding should result in an almost homozygous genetic pattern which would nearly eliminate the antigen specificity of their tissue proteins. Pairs prepared from these animals remain in perfect health for the normal life expectancy of the rat. The wasting and anemia so often described for parabiotic rats are not observed in any of these pairs. Littermates were surgically united by the open coelomic method of Bert (3) at 20 to 30 days of age.

#### DISTRIBUTION OF GROWTH HORMONE IN FLUID SPACES OF THE BODY

In the case of a substance which is confined to the true blood or plasma space of the body, it can be calculated from the flow of blood between the animals that 0.66 per cent of the total present will be transferred to the partner per minute. However, in the case of a substance which is distributed within a larger space, e.g. the total

TABLE I. GROWTH HORMONE CONTENT OF PLASMA<sup>1</sup>

TIME AFTER INJECT. OF GROWTH HORMONE	NO. OF RATS INJECTED	NO. OF ASSAY RATS	TIBIAL CARTILAGE WIDTH OF ASSAY RATS	AV. CARTILAGE WIDTH	GROWTH HORMONE CONTENT/CC. PLASMA
min.			$\mu$	$\mu$	$\mu$
15	4	4	266 267 273 266	268	10.5
30	4	4	247 260 247 247	250	6.2
60	4	4	223 212 193 212	210	2.0

<sup>1</sup> Fifteen, 30 and 60 minutes after intravenous administration of 2.0 mg. of pure growth hormone to 400-gm. hypophysectomized rats.

extracellular fluid, the percentage of the total which will be transferred to the partner per minute will be necessarily smaller. This makes it necessary to determine the distribution of growth hormone in the fluids of the body or what might be called determination of the 'growth hormone space' of the body. This was first approached by injecting known amounts of protein growth hormone, prepared as described by Li, Evans and Simpson (4), intravenously and determining the dilution by extrapolation of the plasma values obtained after various time intervals. Whole blood and plasma samples were assayed by injection into standard assay animals<sup>2</sup>. The growth hormone content of the various samples was determined by comparison of the resulting widths of the proximal tibial cartilage to a standard dose-cartilage response curve for standardized animals, figure 1 (from ref. 5). The dilution experiment was conducted by giving 2.0 mg. of growth hormone in 0.5 cc. of saline into the tail vein of

<sup>2</sup> Female rats of the Long-Evans strain hypophysectomized 26 to 28 days of age and injected daily for 4 days 2 weeks later.

hypophysectomized animals weighing approximately 400 gm. These animals had been hypophysectomized 2 weeks previously. Hematocrits and blood volume studies were made on all animals to show that they did not vary significantly from normal. Fifteen minutes after the injection, the animal was opened and as much blood as could be recovered was drawn from the inferior vena cava into a heparinized syringe. The recovered blood was centrifuged and the plasma decanted; this usually amounted to about 4.0 cc. of plasma. One cc. of this plasma was then injected immediately i.p. into each of 4 standard assay rats and the entire process was repeated for 4 days, a new donor rat to provide the plasma being prepared and killed daily. On the 5th day the test animals were autopsied (96 hours after the first injection) and the proximal tibial cartilage width was measured. A second and third series were done in exactly the same manner except that the time periods after injection of the donor were extended to 30 and 60 minutes. The results are summarized in table 1 and the semi-logarithmic extrapolation is shown in figure 2. The dilution, as determined by extrapolation, is seen to be 18  $\mu\text{g}/\text{cc.}$  of plasma. A 400-gm. rat has a 9.6 cc. plasma volume,

TABLE 2. COMPARISON OF AVERAGE CONCENTRATION OF GROWTH HORMONE IN PLASMA AND LYMPH<sup>1</sup>

DONOR RAT NO.	BODY WEIGHT	PLASMA VOLUME	AV. CONCENTRATION DURING 3-HOUR PERIOD	
			Plasma	Lymph
	gm.	cc.	$\mu\text{g}/\text{cc.}$	$\mu\text{g}/\text{cc.}$
1	150	3.6	25	22
2	215	5.1	18	25
3	174	4.2	21	9
4	208	5.0	18	16
5	130	3.1	29	30

<sup>1</sup> During a 3-hour period after intravenous injection of 5.0 mg. of pure growth hormone.

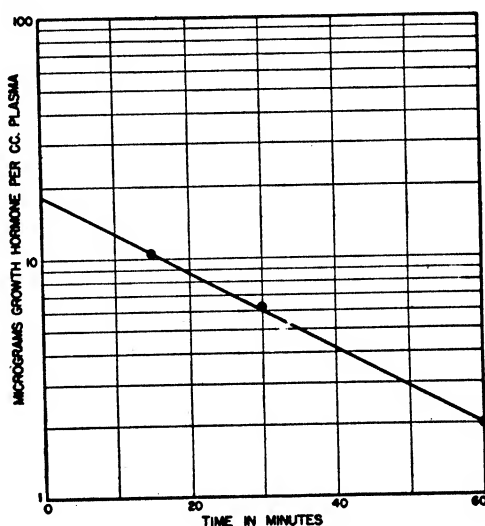
which means 173  $\mu\text{g.}$  or 8.6 per cent of the growth hormone was present in the plasma at zero time. This would indicate that the 'growth hormone space' of the body is much larger than the plasma volume; in fact, it is 28 per cent of the body weight. This suggests that growth hormone would be found in extra cellular fluid in concentrations comparable to those in plasma (none was found on red blood cells).

In order to show the passage of growth hormone into the extracellular fluid compartment of the body, it was decided to collect lymph from the thoracic duct after administration of pure growth hormone in large amounts. Three normal rats ranging in weight from 150 to 215 gms. were prepared with thoracic duct cannulae<sup>2</sup> and after flow from the cannulae had become steady, 5.0 mg. of growth hormone were injected i.v. into the tail vein. The lymph was collected into a beaker submerged in acetone and dry ice so that it was immediately frozen. The lymph was collected over the next 3 hours. Three cc. of this lymph were injected i.p. daily for 4 days into standard assay animals (7 assay animals in all) and the tibial cartilage widths were measured on the 5th day. When compared to the dose-cartilage width response curve

<sup>2</sup> The thoracic duct cannulae were prepared by Miss M. Cleaves of the Department of Anatomy University of California.

(fig. 1) it is found that the average concentration of growth hormone in collected lymph was  $19 \mu\text{g}/\text{cc.}$ , table 2. There was no stimulation of cartilage growth from injections of lymph from normal animals which had not received growth hormone. It is interesting to compare this with the average concentration in the plasma during the 3-hour period following the injection of the growth hormone. It can be seen from the results of the previous dilution problem, figure 2, that the initial quantity in the plasma would be 8.6 per cent of the administered dose or  $430 \mu\text{g.}$  In a 150-gm. rat the plasma volume would be 2.4 per cent of the body weight (6) or 3.6 cc.;  $430 \mu\text{g.}$  distributed in 3.6 cc. gives an initial concentration of  $120 \mu\text{g}/\text{cc.}$  The hormone is removed at these highly unphysiological levels with a biological half-time of 26

Fig. 2. DISAPPEARANCE OF GROWTH HORMONE from the plasma after a single intravenous injection of 2.0 mg.



minutes (50% of the total amount present is removed every 26 minutes, fig. 2). Substituting these values in the formula for average concentration over the 3-hour period gives:

$$\bar{X} = \frac{C_0}{\lambda} [1 - e^{-\lambda t}] = \frac{\frac{120}{.693} [1 - e^{-(.693/26) \times 180}]}{180} = 25 \mu\text{g}/\text{cc.}$$

Where  $\bar{X}$  = average concentration of growth hormone in plasma over time  $t$ .

$C_0$  = initial concentration of growth hormone in plasma.

$\lambda$  = decay constant.

$t$  = time.

This calculation for the 3 donor animals is given in table 2. The average concentration in the plasma during the 3-hour collection period was  $21 \mu\text{g}/\text{cc.}$  and the average concentration in the collected lymph was  $19 \mu\text{g}/\text{cc.}$  Not only was growth hormone

demonstrated in lymph but the concentration was found to be essentially the same in lymph as in plasma.

#### DISTRIBUTION OF GROWTH HORMONE BETWEEN PARABIONTS

The distribution of growth hormone between parabionts was determined by hypophysectomizing both members of pairs 2 weeks after union or at 35 days of age and injecting only one of the members with 220  $\mu\text{g}$ . of pure growth hormone s.c. in 2 injections daily for the next 16 days. It appears from figure 3 that 220  $\mu\text{g}$ . a day has approximately the growth-stimulating potency of the normal pituitary at this

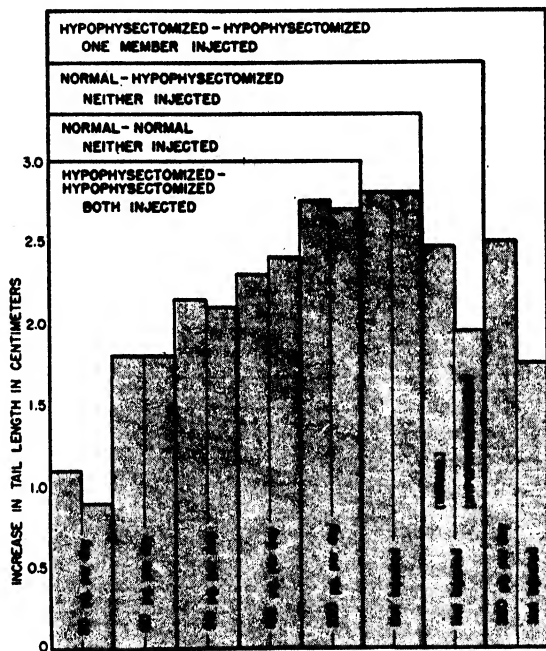


Fig. 3. EFFECT OF GROWTH HORMONE on tail length of parabionts.

age. The increase in the anus-tail-tip length during this 16-day period was used as a quantitative measurement of the amount of growth hormone each partner received by comparison to a standard chart prepared by injecting various doses of growth hormone into both members of hypophysectomized-hypophysectomized parabionts. The increase in anus-tail-tip length of these standard animals is shown in table 3 and figure 3 and the results are plotted on a semi-logarithmic scale in figure 4. By comparing the increase in anus-tail-tip length of the pairs of which only one member received 220  $\mu\text{g}$ /day of pure growth hormone to the standard chart, figure 4, it is seen that the injected members had an average increase in tail length of 2.5 cm. which corresponds to a dose of 150  $\mu\text{g}$ /day on the standard chart. The uninjected members

had an average increase in tail length of 1.75 cm. which corresponds to a daily dose of 55  $\mu\text{g}$ /day (table 4). Their combined growth accounts for 93 per cent of the hormone injected. The uninjected member received 27 per cent of the hormone. The distribution ratio for growth hormone between injected and uninjected members is therefore 2.7 to 1.

In a normal-hypophysectomized uninjected combination the normal animal's tail length increase over the 16-day test period was 2.47 cm. (table 5 and fig. 3) which corresponds on the standard curve to a dose of 140  $\mu\text{g}$ /day and the hypophysectomized partner's tail increased 1.95 cm. or a response equivalent to the injection of 70  $\mu\text{g}$ /day. The combined growth of the pair under the influence of a single pituitary is equivalent to that which would result from the injection of 210  $\mu\text{g}$ . of growth hormone with a distribution between the animals of 2 (normal) to one (hypophysec-

TABLE 3. INCREASE IN TAIL LENGTH OF PARABIONTS<sup>1</sup>

RAT NO.	DAILY DOSE	INCREASE IN ANUS TO TAIL LENGTH	AV. INCREASE IN TAIL LENGTH	RAT NO.	DAILY DOSE	INCREASE IN ANUS TO TAIL LENGTH	AV. INCREASE IN TAIL LENGTH
	$\mu\text{g}$ .	cm.	cm.		$\mu\text{g}$ .	cm.	cm.
59 L	20	1.1	1.0	44 L	120	2.3	2.4
59 R	20	0.9		44 R	120	2.5	
				41 L	120	2.3	
12 L	60	1.8	1.8	41 R	120	2.3	
12 R	60	1.8					
				81 L	220	2.8	2.7
11 L	90	2.2	2.1	81 R	220	3.0	
11 R	90	2.1		79 L	220	2.7	
55 L	90	2.1		79 R	220	2.4	
55 R	90	2.1					

<sup>1</sup> Both members hypophysectomized and both injected daily with pure growth hormone for 16 days after hypophysectomy. Parabiosed at 28 days of age and hypophysectomized at 35 days.

tomized). It is interesting to note that the combined growth of the normal-hypophysectomized pair during this period is approximately equivalent to that of one normal animal (normal-normal, fig. 3). The fact that the pituitary did not increase its production in response to a lowered level of growth hormone during the time of the experiment suggests that the production of growth hormone by the normal pituitary is not under the control of the blood level of growth hormone. The distribution of growth hormone between injected and uninjected parabionts of a hypophysectomized-hypophysectomized pair (2.7:1) and normal-hypophysectomized parabionts (2:1) is not greatly different and may be explained by the synergistic effect of the thyroid hormone and growth hormone in the normal-hypophysectomized pairs. It is known that thyroxin augments or synergizes the effects of growth hormone. For example, 200  $\mu\text{g}$ . of growth hormone plus 5  $\mu\text{g}$ . of thyroxin results in anus-tail-tip increases 75 per cent greater than those achieved with 200  $\mu\text{g}$ . of growth hormone alone (7). For this reason the normal-hypophysectomized parabionts were not believed to give a true



picture of the turnover of growth hormone alone and the calculations are based on the 2.7 to 1 ratio of injected to uninjected (hypophysectomized-hypophysectomized) partners.

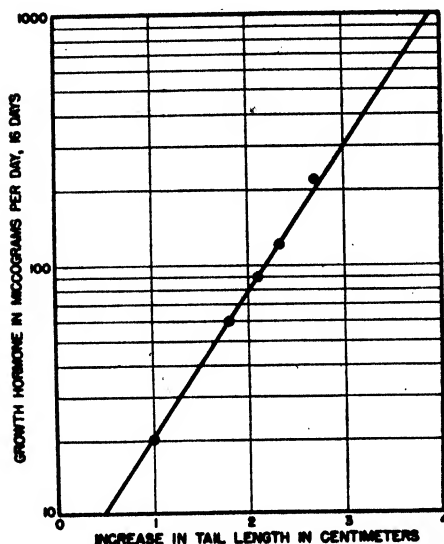


Fig. 4. EFFECT OF GROWTH HORMONE on tail length of hypophysectomized parabionts.

In the case of growth hormone  $1/S = 2.7$ .

$$\frac{a}{V_h} = \frac{a}{V_b} \times \frac{V_b}{V_h} = .0066 \times \frac{4.6}{28} = .00108$$

Where  $V_b$  is the blood volume.

$$\therefore T_r = \frac{1}{.00108 (2.7 - 1)} = 544 \text{ minutes}$$

#### THE DISTRIBUTION OF ADRENOCORTICOTROPHIC HORMONE IN FLUID SPACES OF THE BODY

Greenspan, Li and Evans (8) have shown that the space dilution or 'hormone space' of ACTH may be determined by following the disappearance of i.v. administered ACTH and extrapolating these experimental values to zero time for the dilution volume. After a single i.v. injection of 5.0 mg. of protein ACTH the plasma level fell in an exponential fashion with a biological half-time of disappearance, at these highly unphysiological levels, of 5.5 minutes. The extrapolated value for the level of ACTH at zero time indicates that only 6 per cent of the injected dose is present in the plasma after mixing. This would represent a dilution space equal to 43 per cent of the body weight if equally distributed.

#### CALCULATION OF RATE OF REMOVAL OF GROWTH HORMONE

1) The distribution of growth hormone between parabionts (injected-uninjected) is 2.7 to 1. 2) The rate of exchange of blood between the members is 0.66 per cent of the blood volume/minute. 3) The 'growth hormone space' of the body is 28 per cent of the body weight. And 4) the rate of removal of growth hormone from the circulation will be found by solution of the following equations:

Define  $S \equiv \frac{C_{\bar{h}}}{C_N}$

Equation 1 can be rearranged to give the average life ( $T_r$ ) in terms of  $S$  explicitly as:

$$T_r = \frac{1}{\frac{a}{V_h} (1/S - 1)} \quad (2)$$

Such a great dilution of i.v. administered ACTH suggests that it is widely distributed in the fluids of the body and would be found in lymph and extracellular fluid. To demonstrate the passage of ACTH into lymph, a 260-gm. rat was prepared with a thoracic duct cannula, and after flow from the cannula was steady, 7.0 mg. of ACTH was injected in one cc. into the tail vein and the lymph was collected for the next hour. The collected lymph was injected into standard assay animals (one cc. twice a day for 4 days, or a total of 8 cc. were injected). Lymph from uninjected donors was injected into standard animals as a control. The assay animals were autopsied 96 hours after the first injection and the adrenals were weighed and examined

TABLE 4. EFFECT OF GROWTH HORMONE ON TAIL LENGTH OF PARABIONTS<sup>1</sup>

PAIR NO.	GROWTH HORMONE INJECTED	INCREASE IN ANUS TAIL TIP LENGTH	AV. INCREASE IN TAIL LENGTH	PAIR NO.	GROWTH HORMONE INJECTED	INCREASE IN ANUS TAIL TIP LENGTH	AV. INCREASE IN TAIL LENGTH
	$\mu\text{g/day}$	cm.	cm.		$\mu\text{g/day}$	cm.	cm.
78 L	220	2.60	2.50	78 R	none	1.80	1.75
75 L	220	2.40		75 R	none	1.60	
76 L	220	2.35		76 R	none	1.70	
21 L	220	2.60		21 R	none	1.90	

<sup>1</sup> Both members hypophysectomized at 35 days of age but only one member injected with 220  $\mu\text{g.}$  of pure growth hormone daily for 16 days after hypophysectomy.

TABLE 5. INCREASE IN TAIL LENGTHS OF NORMAL-HYPOPHYSECTOMIZED PARABIONTS<sup>1</sup>

PAIR NO.	TYPE OF ANIMAL	INCREASE IN ANUS-TAIL TIP LENGTH	AV. INCREASE IN TAIL LENGTH	PAIR NO.	TYPE OF ANIMAL	INCREASE IN ANUS-TAIL TIP LENGTH	AV. INCREASE IN TAIL LENGTH
		cm.	cm.			cm.	cm.
52 R	Normal	2.60	2.47	52 L	Hyph'd	2.00	1.95
99 R	Normal	2.40		99 L	Hyph'd	1.95	
79 R	Normal	2.40		79 L	Hyph'd	1.90	

<sup>1</sup> Initial measurements and hypophysectomy at 35 days of age, final measurement 16 days later.

histologically. The average adrenal weight was 11 mg. in the experimental as compared to 9 mg. in the control group. The histology was characterized by slightly wider cortex, obliteration of subglomerular sudanophobic zone, lipid distribution almost uniform and reappearance of the *zone reticularis* (fig. 6). The histological changes were thought to correspond to a total dose of 50 to 100  $\mu\text{g.}$  The entire dose was administered in 8 cc. of lymph which would mean an ACTH content of from 6 to 12  $\mu\text{g./cc.}$  of collected lymph. It was shown by Greenspan, Li and Evans (8) that the initial concentration after i.v. administration of ACTH is 6 per cent of the injected dose and at these high levels the hormone is removed with a biological half-time of 5.5 minutes. Thus a 260-gm. rat receiving 7 mg. would have an initial plasma concentration of ACTH of (420  $\mu\text{g.}$  in 6.2 cc. plasma or 68  $\mu\text{g./cc.}$ ) 68  $\mu\text{g./cc.}$  The

average concentration of ACTH in the plasma over the one-hour collection period would be:

$$\bar{X} = \frac{\frac{C_0}{\lambda} [1 - e^{-\lambda t}]}{t} = \frac{\frac{68}{5.5} [1 - e^{-(.693/5.5) \times 60}]}{60} = 9 \mu\text{g/cc.}$$

Therefore not only was ACTH demonstrated in lymph but was found in essentially the same concentration as in plasma.

TABLE 6. COMPARISON OF ADRENAL WEIGHTS OF MALE RATS IN PARABIOSIS

NO. OF RATS	GROUP			
	1 <sup>1</sup>	2	3 <sup>1</sup>	4
	Hyp'h'd	Normal	Hyp'd-Hyp'h'd	Normal-Normal
	mg.	mg.	mg.	mg.
1	7.4	18.0	5.8	15.0
2	6.5	16.5	3.0	17.0
3	6.3	13.5	5.0	14.5
4	6.7	13.0	4.6	15.0
5	6.0	17.0	5.3	11.2
6	7.4	15.5	5.0	12.0
7	5.0	13.5	5.6	13.5
8	5.9	12.0	4.5	15.5
9	6.5	13.0	4.5	15.5
10	6.5	15.0	5.0	12.2
11			5.0	16.0
12			4.2	15.0
Av.....	6.3±0.23 <sup>2</sup>	14.7±0.63	4.8±0.21	14.3±0.51

Age at parabiosis, 28 days; age at hypophysectomy, 75 days; age at autopsy, 105 days.

<sup>1</sup> Test for significance of stimulation of hypophysectomized animals' adrenals by parabiosis to normal partner (comparison of groups 1 and 3),  $P < 0.001$  (9). <sup>2</sup> Standard error of mean.

#### DISTRIBUTION OF ADRENOCORTICOTROPHIC HORMONE BETWEEN PARABIOTANTS

The distribution of adrenocorticotrophic hormone between parabionts was determined by comparing the adrenal weights of hypophysectomized members with those of their normal partners. Male Slonaker rats were parabiosed at 28 days of age, one member hypophysectomized at 75 days and the pair autopsied for adrenal weights at 105 days of age. The average weight of the adrenals of the normal members was 14.7 mg., and the adrenals of the hypophysectomized members had an average weight of 6.3 mg. Thus the adrenals of the normal partners weighed 2.3 times as much as those of the hypophysectomized partners (table 6). The adrenal weights of the normal members are the same as those of normal-normal parabionts. However the average weight of the adrenals from hypophysectomized-hypophysectomized parabionts is 4.8 mg. which is significantly under the 6.3 mg. average for the hypophysectomized animals which were in parabiosis with normal partners,  $P < 0.001$

(9). By comparing these weights to the standard dose-adrenal response curve of figure 6 which was prepared by injecting various doses of adrenocorticotrophic hormone into hypophysectomized rats,<sup>4</sup> it is seen that an adrenal weight of 6.3 mg.



Fig. 5. ADRENAL RESPONSE FROM LYMPH of animals injected with ACTH demonstrating passage of ACTH into lymph.

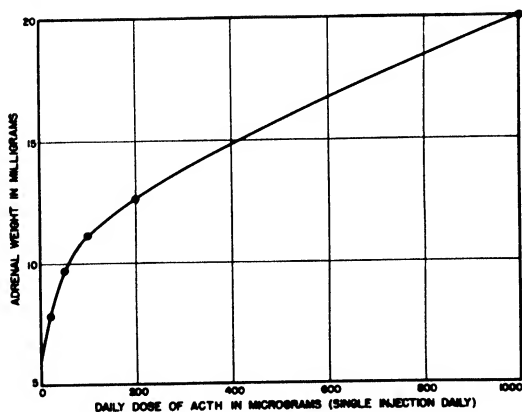


Fig. 6. BIOASSAY OF ADRENOCORTICOTROPIC HORMONE in male rats hypophysectomized at 40 days (maintenance test).

would result from the injection of 5.0 µg. of adrenocorticotrophic hormone a day, and an average adrenal weight of 14.7 mg. would require the injection of 425.0 µg. a day.

<sup>4</sup> Prepared from data presented by Li, Evans and Simpson (10).

Therefore the distribution of adrenocorticotrophic hormone between parabionts is 85 (the producing animal) to 1 (the non-producing animal).

#### CALCULATION OF RATE OF REMOVAL OF ADRENOCORTICOTROPHIC HORMONE

It is now possible to substitute experimental values for all of the functions in the equation derived to explain the distribution of substances between parabionts except the rate of removal of adrenocorticotrophic hormone at physiological concentrations, for which we can now solve: 1) The distribution of adrenocorticotrophic hormone between normal-hypophysectomized parabionts is 85 to 1. 2) The rate of exchange of blood between the members is 0.66 per cent of the blood volume per minute. 3) The 'adrenocorticotrophic hormone space' of the body is 43 per cent of the body weight, and 4) the rate of removal of adrenocorticotrophic hormone from the circulation will be found by solution of the following equation:

$$T_r = \frac{1}{\frac{a}{V_h} (1/S - 1)} \quad (2)$$

$$\frac{a}{V_h} = \frac{a}{V_b} \times \frac{V_b}{V_h} = .0066 \times \frac{4.6}{43} = 7 \times 10^{-4}$$

$$\therefore T_r = \frac{1}{7 \times 10^{-4} (85 - 1)} = 17 \text{ minutes}$$

In considering the results obtained from calculations of this sort for either growth hormone or ACTH, one must realize that the final value includes the propagated error from the possible errors of each of the determinations involved. Only the magnitude of the final value is of importance when the calculations depend, as in one case, on the use of as rough a quantitative estimation as the comparison of adrenal weights. Methods being studied at the present time for direct assay of plasma samples for ACTH activity would improve the accuracy of the determination of the distribution of ACTH between parabionts.

#### CONCLUSIONS

Growth hormone and adrenocorticotrophic hormone leave the blood vascular channels and become distributed in the larger volume of the extra cellular fluid of the body. The 'growth hormone space' of the body is equivalent to 28 per cent of the body weight. The 'adrenocorticotrophic hormone space' of the body is equivalent to 43 per cent of the body weight. On the basis of the data presented, the average life of the circulating growth hormone molecule at physiological levels in the rat was calculated to approximate 9 hours. From similar data the average life of the adrenocorticotrophic hormone molecule in the circulation of the rat at physiological levels was approximately 17 minutes.

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# ANTI-INSULIN AND DIABETOGENIC ACTIONS OF PURIFIED ANTERIOR PITUITARY GROWTH HORMONE<sup>1,2</sup>

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THE mechanism of insulin hypersensitivity characteristic of hypophysectomized animals is still poorly understood. In earlier studies the effects of thyroxin (1) and adrenal cortical extract (2) were investigated. While each of the above-mentioned hormones (when given in large doses) produced an anti-insulin action, neither was able to revert the insulin response of the hypophysectomized dog to the type seen in a normal animal. We next turned our attention to those anterior pituitary hormones which are believed to act without the mediation of other endocrine glands. The first of these hormones to be investigated was purified growth hormone. The results obtained during the course of the study of the effect of growth hormone on insulin hypersensitivity led us to extend the investigation to include the diabetogenic activity of this hormone. The present report is concerned with the effect of purified anterior pituitary growth hormone on the insulin hypersensitivity and glucose tolerance of hypophysectomized dogs.

## METHODS

All experiments were carried out on trained normal and hypophysectomized dogs. Both male and female animals were used. The hypophysectomies<sup>4</sup> were performed by the oral approach described in an earlier work (3). Following removal of the hypophysis, only those animals were used that exhibited maximal insulin hypersensitivity, i.e. their response to insulin was 60 to 100 times greater than that occurring in normal animals (4). In our experience this degree of hypersensitivity to insulin has been a reliable indication of successful removal of the pituitary gland. However, we have considered hypophysectomies complete only if examination of serial sections of a block, consisting of the tissue occupying the region of the sella turcica with the overlying brain tissue, has failed to reveal any pituitary cells. As

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<sup>2</sup> This work was presented before the American Physiological Society at Atlantic City, April, 1950. A preliminary report of part of this work appeared in the *Federation Proc.* 9: 30, 1950. A brief account of this work appeared in *Proc. Soc. Exper. Biol. & Med.* 74: 524, 1950.

<sup>3</sup> Dazian Foundation Fellow.

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an additional criterion of the completeness of the hypophysectomy, we have also required that the thyroids, adrenals, and the gonads show the characteristic atrophic changes (3). The completeness of the removal of the pituitary gland in each of the animals used in this study will be judged by application of the aforementioned criteria. Gross and microscopic studies will also be made of various organs with special attention to the pancreas. These studies are being performed by Dr. Nathan Lane, University Hospital, New York University-Bellevue Medical Center, and will be published at a later date.

The animals were maintained on a mixed diet. The basic diet for a 10 kg. dog consisted of 75 gm. cracker meal, 30 gm. skimmed milk powder, 30 gm. powdered meat (liver) residue, 10 gm. brewer's yeast, 5 cc. cod liver oil, 17 cc. corn oil, 3 gm.

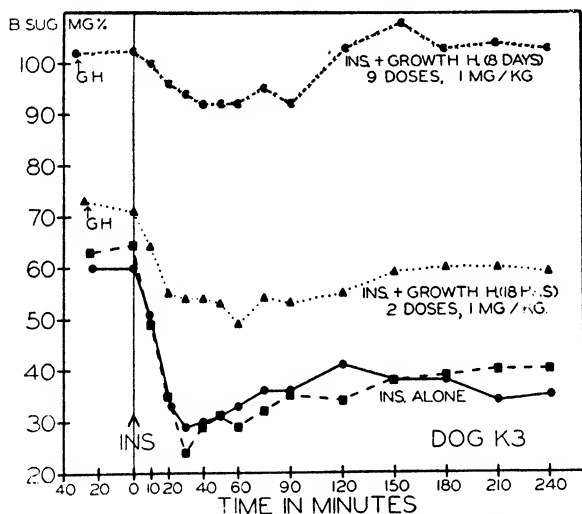


Fig. 1. EFFECT OF INSULIN (INS), 0.025 unit/kg., given intravenously on blood sugar (B SUG.) of hypophysectomized (Hyphex) dog K3 before and during growth hormone (G.H.) treatment (2 doses and 9 doses).

salt mixture (sodium chloride, potassium phosphate, magnesium citrate, and ferric citrate). This diet, mixed with water, provided 45 gm. protein, 74 gm. carbohydrate, and 34 gm. fat, and represented approximately 785 calories. The basic food allotment was periodically adjusted to maintain a constant body weight. In most instances the diet was divided and the animals fed twice daily. On occasion, when a meal was rejected, approximately equicaloric amounts of meat, fish, or milk were substituted. Water was provided *ad libitum*.

Experiments were performed only on animals that ate regularly and that consumed their full food allotment on the day preceding the experiment. The experiments were started in the post-absorptive state, 17 to 18 hours after the last feeding. No anesthetic agent was used.

The response to the hypoglycemic action of insulin was determined by the



intravenous (i.v.) administration of 0.025 unit of insulin (Lilly)<sup>5</sup> per kg. body weight, unless otherwise stated. Hereafter, this dose of insulin will be referred to as the test dose. The glucose tolerance test was performed by the i.v. administration of 0.075 gm. glucose/kg. body weight/minute for 10 minutes. In both instances venous blood samples for sugar determinations were taken at frequent, stated intervals for a period of 4 hours. Blood sugar was determined by the Hagedorn-Jensen method (5) using the Somogyi acid-zinc filtrate (6).

The growth hormone, which was supplied by Armour Laboratories,<sup>6</sup> (lot # 22KR1) contained approximately 85 per cent of an electrophoretically homogeneous component which had a mobility comparable to that of pure growth hormone. Assay of this material in the Armour Laboratories showed that 50 gamma daily were

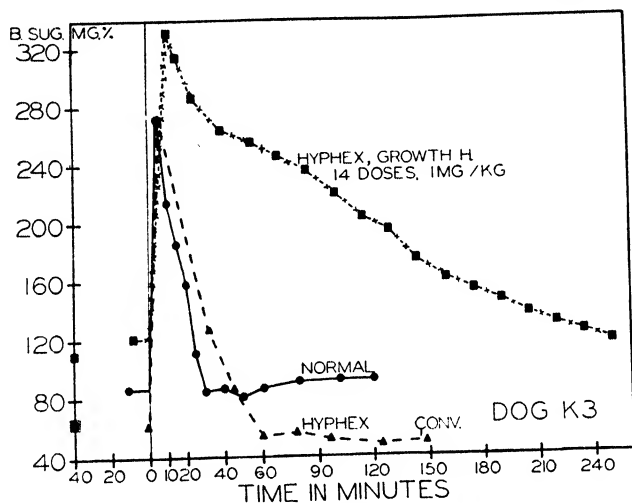


Fig. 2. INTRAVENOUS GLUCOSE tolerance test (0.075 gm. glucose/kg/min. for 10 minutes) Blood sugar curves of normal and hypophysectomized dog K3 before and after growth hormone treatment (14 doses).

sufficient to cause a gram per day weight increment in a 50 to 60 gm. hypophysectomized rat. This preparation contained thyrotropic hormone, the activity of which was equivalent to 1.2 per cent of Armour's Standard Thyrotropin. It also contained lactogenic, adrenocorticotrophic and posterior pituitary hormones. Its lactogenic activity was estimated to be less than 5 per cent; its adrenocorticotrophic activity less than one per cent of Armour ACTH Standard La-I-A; and its posterior pituitary activity between 0.005-0.01 unit/mg. The dosage, routes and circumstances of administration will be described below.

#### RESULTS

*Effects of Growth Hormone on Insulin Hypersensitivity in Hypophysectomized Dogs.* In 18 normal dogs (37 experiments) the test dose of insulin (0.025 unit/kg.)

<sup>5</sup> Insulin was kindly supplied by Dr. K. K. Chen of Eli Lilly & Co.

<sup>6</sup> We are greatly indebted to Drs. E. E. Hays and I. M. Bunding of Armour Laboratories for their generous supply of growth hormone.

produced either no change or a slight drop in blood sugar which returned to normal within 40 minutes. Administration of the test dose to 37 hypophysectomized dogs (111 experiments) resulted in a precipitous fall in blood sugar to very low levels (4). In some animals the blood sugar remained at a very low level throughout the experimental period, as for example in *dog K3* (fig. 1). In others, the blood sugar tended to rise slowly during the course of 4 hours, for example in *dog K8* (fig. 3). In normal dogs, a dose of 1.5 units of insulin/kg. (i.e. 60 times the test dose) given i.v. was required (fig. 5) in order to produce a response similar to that which occurred in the hypophysectomized *dog K8* after the test dose of insulin (fig. 3). The effect noted in other hypophysectomized dogs, for example in *K3* (fig. 1), was observed in normal animals only after the administration of 2.5 units/kg. of insulin (i.e. 100 times the

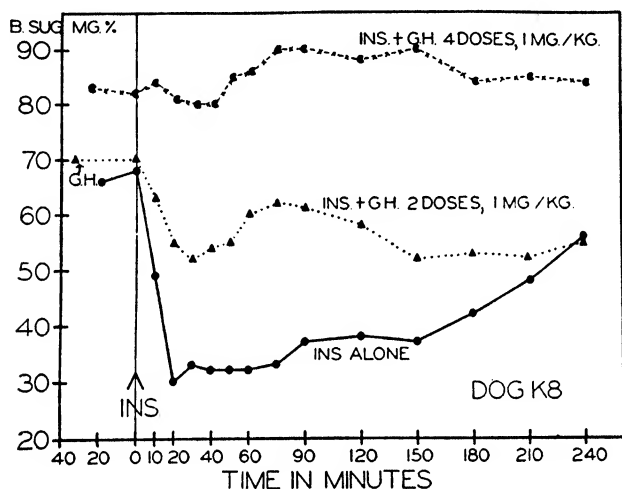


Fig. 3. EFFECT OF INSULIN, 0.025 unit/kg., given intravenously on blood sugar of hypophysectomized *dog K8* before and during growth hormone treatment (2 doses and 4 doses).

test dose). Our estimate that the hypophysectomized dogs were 60 to 100 times more sensitive to insulin than normal dogs was based on a large number of similar experiments.

During the course of 6 growth hormone studies performed on 4 hypophysectomized dogs, 36 insulin sensitivity tests were done. The administration of growth hormone greatly modified the hypophysectomized dog's response to insulin. In one series of experiments, the effects of two doses of growth hormone were studied. The first dose of growth hormone, 1 mg/kg., was administered intramuscularly and the animals were fed immediately afterwards. Eighteen hours later the same dose of growth hormone was administered i.v. to these animals in the post-absorptive state. A slight rise in blood sugar was apparent in some animals prior to the administration of this second dose of growth hormone (fig. 1). The insulin sensitivity test was begun 30 minutes after the second injection of growth hormone. No change was observed in the blood sugar level between the second dose of growth hormone and the administration of insulin (figs. 1, 3). The response to the test dose of insulin was significantly

diminished. The blood sugar showed a less marked fall and never reached the low levels observed in the experiments before growth hormone treatment. However, despite this less pronounced fall, it is noteworthy that the blood sugar did not rise to the post-absorptive blood sugar level during the experimental period (figs. 1, 3).

Continued daily administration of the same dose of growth hormone completely abolished the exaggerated response to insulin. After 4 doses of growth hormone the post-absorptive blood sugar was distinctly elevated and no fall in blood glucose occurred following the administration of the test dose of insulin (fig. 3). The results observed after 9 doses of growth hormone can be seen in figure 1. At this time the post-absorptive blood sugar was far above the normal range and again insulin produced no significant change.

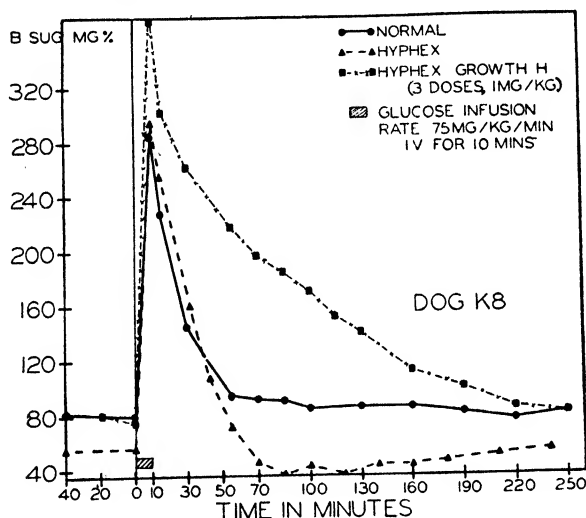


Fig. 4. INTRAVENOUS GLUCOSE tolerance test. Blood sugar curves of normal, and of hypophysectomized dog K8 before and after growth hormone treatment (3 doses).

*Effect of Growth Hormone Administration on Glucose Tolerance in Hypophysectomized Dogs.* Untreated hypophysectomized dogs have a post-absorptive blood sugar level somewhat lower than normal, however, their response to intravenous glucose was similar to that seen in normal animals except that there was always a marked hypoglycemic phase following the initial period of hyperglycemia (figs. 2, 4). In contrast, hypophysectomized dogs receiving daily administrations of growth hormone had elevated post-absorptive blood sugar levels and their intravenous glucose tolerance tests revealed marked impairment of glucose utilization indicative of a diabetic state (figs. 2, 4). Slight impairment of the tolerance to glucose was noted as early as 18 hours after the first injection of growth hormone (i.e. after a total of 2 doses). As can be seen in table 1, the peak of the blood sugar curve was higher, the return to the normal fasting level significantly delayed, and the hypoglycemic phase practically abolished. Neither glycosuria nor acetonuria was noted even after 3 weeks of

continued growth hormone treatment of 1 mg/kg/day. When growth hormone treatment was discontinued both the response to insulin and the response to intravenous glucose gradually approached those observed prior to growth hormone administration. However, in one dog treated with the hormone for 3 weeks (1 mg/kg/day), a reversion to the original response to insulin and to glucose did not occur even after 3½ months following the cessation of treatment.

*Development of Insulin Resistance in Hypophysectomized Dogs.* After a series of injections of 1 mg/kg/day of growth hormone, the hypophysectomized animals not only lost their hypersensitivity to insulin but even showed less response to large doses of insulin than did the untreated normal animals. As can be seen in figure 5, 1.5 units/kg. of insulin (i.e. 60 times the test dose) produced less effect on the blood sugar level of growth hormone treated hypophysectomized dogs than it did on that of untreated normal animals. Thus it is evident that resistance to insulin developed after prolonged growth hormone treatment in the hypophysectomized dogs.

TABLE 1. INTRAVENOUS GLUCOSE TOLERANCE TEST (0.075 GM. GLUCOSE/KG./MIN. FOR 10 MIN.) IN HYPOPHYSECTOMIZED DOG K10 BEFORE AND AFTER 2 DOSES OF GROWTH HORMONE

M	0	10	15	30	35	40	55	70	85	100	120	130	190	250
A	62	305	246	142		110	67 <sup>1</sup>	46	44	43	49	50	56	63
B	72	351			183		142	114	96	78	69 <sup>1</sup>	66	71	70

M, time in minutes. A, blood sugar values—mg/100 cc. of blood, before growth hormone. B, blood sugar values—mg/100 cc. of blood, after growth hormone (2 doses, 1 mg/kg.).

<sup>1</sup> Denotes the blood sugar approximating the preinfusion values. Glucose infusion started at 0 time.

#### DISCUSSION

As stated above, the growth hormone preparation used in our experiments contained small amounts of thyrotropic and adrenocorticotrophic hormones. Ingle (7), Conn (8) and their colleagues have demonstrated that the administration of massive doses of adrenocorticotrophic hormone produces an insulin-resistant diabetes. Similar results follow the administration of very large doses of 11-oxycorticosteroids (7). The aforementioned findings as well as previous work done in this laboratory on hypophysectomized dogs indicate that the effects produced with this growth hormone preparation cannot be attributed to the presence of the tropic hormones. Thyroxin (1) and adrenal cortical extract (2) exerted an anti-insulin action only if administered in very large doses and, even then, the action never resembled that produced by the growth hormone preparation.

Thus we conclude, from the data presented, that purified growth hormone exerts a potent anti-insulin action. When growth hormone was administered to the hypophysectomized dog the exaggerated insulin response was first diminished, then abolished and, if the administration of growth hormone was continued for a sufficiently long period, a resistance even to large doses of insulin developed. In the experiments just described a diabetic state accompanied the loss of insulin hypersensitivity.

Several investigators have offered evidence which suggested that purified growth hormone possesses diabetogenic activity. Evans and his colleagues (9) demonstrated that a purified growth hormone preparation increased the glycosuria of sucrose-fed partially depancreatized rats. Later from the same laboratory, Bennett and Li (10) reported that growth hormone increased the glycosuria in some cases of alloxan-diabetic rats. Milman and Russell (11) observed a rise in blood sugar in both alloxanized and partially depancreatized rats following the administration of purified growth hormone. While our investigation was in progress, two reports appeared in which it was shown that purified growth hormone could produce diabetes. Young and his colleagues (12) noted glycosuria in normal cats following growth hormone administration. Houssay and Anderson (13) were able to induce diabetes in dogs and cats after 85 and 74 per cent respectively of the pancreas had been removed prior to the

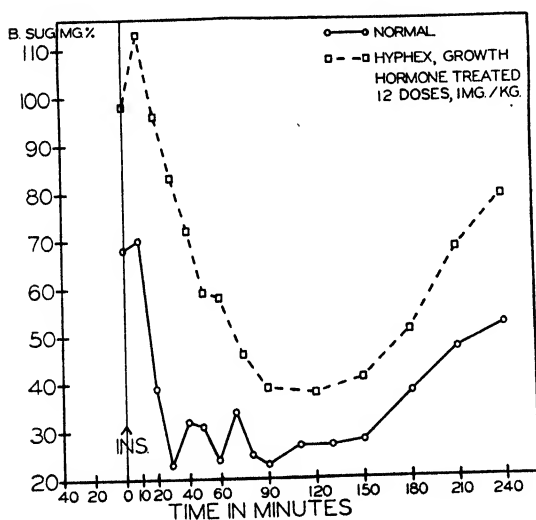


Fig. 5. COMPOSITE BLOOD SUGAR curve produced by insulin, 1.5 units/kg., in 13 normal dogs compared with the blood sugar curve produced by insulin, 1.5 units/kg., in growth hormone treated (12 doses) hypophysectomized dog.

hormone treatment. For the former species 50 mg/kg. of growth hormone was the daily dose and for the latter, 25 mg/kg. To date, there have been no reports dealing with the effects of purified growth hormone on insulin response whereas the major part of our investigation is concerned with just this problem. The hypophysectomized animal (especially the dog), with its great hypersensitivity to insulin, appears to be particularly well suited for the study of the anti-insulin action of growth hormone.

The insulin hypersensitivity of hypophysectomized animals has never been satisfactorily explained. Any factor or a combination of several factors may be involved, namely defective gluconeogenesis, impaired glycogen mobilization, retarded inactivation of insulin or increased sugar utilization. Thus, an agent which is capable of modifying the insulin hypersensitivity of hypophysectomized animals may act on any one or any combination of these factors. In analyzing the anti-insulin action of growth hormone therefore, it is necessary to examine the effects of this hormone on the aforementioned phenomena.

In our experiments (performed on hypophysectomized animals with evidence

of adrenal cortical atrophy) increased gluconeogenesis cannot be entertained as a factor responsible for the growth hormone induced anti-insulin action. It has been well established by Evans and his colleagues that growth hormone produces nitrogen retention and lowers the blood amino-nitrogen (14). A decrease of available glucose precursors (amino acids) should result, thereby decreasing gluconeogenesis. There is no evidence available that the primary action of growth hormone is to increase glycogen mobilization. Increased inactivation of insulin by growth hormone is also without experimental support. There remains then the possibility that growth hormone affects the peripheral utilization of carbohydrate consequently altering insulin hypersensitivity. The impairment of glucose tolerance evident in our growth hormone treated animals is indicative of defective carbohydrate utilization. Moreover, evidence has been presented by Stadie *et al.* (15) and Park *et al.* (16, 17) that purified growth hormone had an inhibitory influence on carbohydrate utilization in the isolated rat diaphragm provided that the hormone was injected into the living animal.

The results of our experiments suggest that growth hormone exercises a physiological control of peripheral utilization of carbohydrate. This hypothesis is further supported by the evidence that hypophysectomized rabbits (18) and rats (19, 20), and the isolated diaphragms of hypophysectomized rats (21-23) exhibit increased sugar utilization. Thus the lack of growth hormone, which seems to be at least partially responsible for the increase in sugar utilization in hypophysectomized animals, appears to be an important factor in producing the hypersensitivity to insulin. It follows then that the administration of growth hormone to hypophysectomized animals decreases sugar utilization consequently accounting for the decreased response to insulin. The observed diabetic state is considered to be the result of an exaggeration of the physiological action of growth hormone on peripheral utilization of carbohydrate. We have reason to believe that a further reduction in the dose of growth hormone will exert an anti-insulin action without producing diabetes.

Although the growth hormone induced diabetes is in all likelihood due to a decreased peripheral utilization of carbohydrate, it probably becomes apparent only when the islets of Langerhans no longer are capable of adjusting (by increasing insulin output) to this alteration in carbohydrate metabolism. It has been demonstrated (Houssay (24), Young (25), Best (26), and their colleagues) that prolonged administration of growth promoting anterior pituitary extracts to normal dogs produced degeneration of the islet cells. These observers postulated that the degeneration was the result of excessive stimulation of the islets, the latter phenomenon being due either to a direct action of the anterior pituitary extracts or to an indirect one resulting from a sustained hyperglycemia. That persistent elevation of blood sugar can damage the islet cells was shown by Dohan and Lukens (27). It is not unlikely that in the hypophysectomized dog purified growth hormone can also lead to an exhaustion of the insulin secreting cells by means of either a direct or an indirect mechanism.

#### SUMMARY

Purified anterior pituitary growth hormone exerts a very potent anti-insulin action in hypophysectomized dogs, i.e. it diminishes and, on continued administra-

tion, abolishes the exaggerated hypoglycemic response to insulin. Growth hormone (in the dosages used) produces a diabetic state in hypophysectomized dogs as evidenced by their impaired tolerance to intravenous glucose. Alteration of the insulin response by growth hormone (in the regimen used) is accompanied in hypophysectomized dogs by a concomitant diabetic state. After prolonged administration of growth hormone, the hypophysectomized dogs develop a resistance to exogenous insulin. They show less response to large doses of insulin than do untreated normal dogs. It is proposed that the anti-insulin and diabetogenic actions of growth hormone are due to the inhibition of peripheral utilization of carbohydrate. The growth hormone induced diabetes is considered to be the result of an exaggeration of the physiological action of this hormone and probably becomes apparent when a relative deficiency of insulin production develops.

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# ACTION OF CORTISONE ON CARDIOVASCULAR-RENAL EFFECTS OF DESOXYCORTICOSTERONE ACETATE<sup>1</sup>

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**D**URING recent years a considerable amount of information has accumulated (1-3) concerning the hypertensive effects of desoxycorticosterone acetate (DCA). Recently, we have found that not only do small amounts of DCA elicit a rise in blood pressure, but that after discontinuation of a short period of intensive treatment, a sustained elevation in the blood pressure persists in some of the animals (4, 5). In view of these observations and their possible implications in essential hypertension in man it has become of importance to determine whether or not these hypertensive effects of the steroid can be inhibited by related compounds.

We have recently devised a test to screen compounds rapidly for their possible DCA-inhibiting effects (6). In brief, this test consists of the administration of DCA pellets in amounts which cause a discernible elevation in blood pressure in about 2 weeks in male rats of approximately 70 gm. Despite the brief duration of the elevated pressure, an increase in heart weight is consistently observed. Similarly, a considerable increase in renal mass is also noted. Of the first 8 substances which we tested, only one, Lipo-Adrenal Cortex, a potent cortical extract, was in any way capable of inhibiting the cardiovascular-renal effects of DCA. This positive effect of Lipo-Adrenal Cortex has been confirmed by more prolonged experimentation as well.

In view of the known effects of cortisone it seemed logical to test the effects of this compound by the screening method. Because of the considerable importance of cortisone in its own right, and the desirability of accumulating basic data concerning its effects, this communication is concerned not only with its action in DCA-treated animals, but also with some of its effects in the intact rat. In addition to a study of the cardiovascular-renal effects, the study included the determination of sodium, potassium and chloride in plasma at the conclusion of treatment as well as the histological examination of various tissues.

## EXPERIMENTAL

Forty male albino rats of the Sherman strain, weighing between 60 and 70 gm., were divided into 5 groups of 8 animals each. *Group 1* served as untreated control,

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while *group 2* received daily injections of cortisone. The animals of *group 3* received subcutaneous implants of DCA pellets, while those of *group 4* received DCA in similar amounts, together with daily injections of cortisone. As control for the vehicle in which the cortisone was administered, the animals of *group 5* received subcutaneous implants of DCA together with daily injections of the vehicle.

Cortisone acetate was injected as 0.2 cc. twice daily of a saline suspension of microcrystals with added suspending agents and 1.5 per cent benzyl alcohol as preservative (Merck). Each cc. of the preparation contained 5 mg. of cortisone acetate, so that each animal received 2 mg. daily. The control material used in *group 5* of this experiment consisted of the liquid vehicle described above, without the cortisone. DCA was administered in the form of pellets, one-quarter of a 75-mg. Cortate pellet implanted subcutaneously—2 on the first day and one on the 4th, 8th and 12th days of the experiment.

The experiment was continued for 20 days, at the end of which time the animals were killed and numerous tissues taken for histological examination.

Blood pressure, using a modification of the tail plethysmographic method with ether (7), was determined daily beginning on the 13th day of the experiment and continuing through to termination. On the 20th day, blood was obtained by heart puncture without anesthesia for electrolyte determinations on individual samples from each animal (8). Immediately following this, the animals were killed.

The results obtained are tabulated in table 1.

#### *Body Weight*

All animals receiving cortisone showed a complete suppression of growth. This effect was in no way inhibited by the simultaneous administration of DCA. The suppression of growth observed in these small animals cannot be considered entirely innocuous since one animal in *group 2* and two in *group 4* succumbed during the course of the experiment, while no deaths occurred in any of the other groups not receiving cortisone.

#### *Cardiovascular-renal Effects*

The blood pressure values obtained on the last 7 days of the experiment are presented graphically in figure 1. No change in blood pressure occurred in *group 2*, receiving cortisone alone. As usual, DCA caused a progressive significant elevation in blood pressure, observed in both *group 3* receiving DCA alone and in *group 5* receiving DCA together with the aqueous vehicle.

In contrast, cortisone appeared to inhibit the blood pressure elevation caused by DCA. Since these animals did not grow normally, however, this result must be accepted with caution, since it probably does not represent a specific antagonism of DCA by cortisone (see below).

#### *Organ Weights*

Organ weights are expressed per 100 cm.<sup>2</sup> of body surface. Heart weight was, as usual, increased in DCA-treated animals. In the DCA-cortisone group, heart weight was similarly increased, despite the failure of the blood pressure to rise. This

TABLE 1

GROUP NO.	TREATMENT	1		2		3		4		5	
		UNTREATED		CORTISONE		DCA		DCA + CORTISONE		DCA + CORTISONE VEHICLE	
	Initial wt., gm.	65 ± 5		63 ± 6		64 ± 6		65 ± 5		68 ± 5	
	Final wt., gm.	113 ± 10		69 ± 11		130 ± 17		64 ± 8		125 ± 13	
	Blood Pressure, mm. Hg,										
	13th day	90 ± 5		77 ± 13		100 ± 16		81 ± 10		104 ± 14	
	14th day	90 ± 9		79 ± 17		101 ± 15		81 ± 12		97 ± 13	
	15th day	106 ± 16		98 ± 12		125 ± 14		95 ± 18		117 ± 13	
	16th day	111 ± 6		103 ± 13		134 ± 9 S		95 ± 21		124 ± 9 S	
	17th day	113 ± 10		108 ± 8		136 ± 12 S		98 ± 26		125 ± 12 S	
	18th day	114 ± 7		105 ± 14		128 ± 19		97 ± 22		123 ± 14	
	20th day	103 ± 10		105 ± 18		141 ± 5 S		90 ± 20		122 ± 9 S	
	Av.	104		97		123 S		91		116 S	
	Heart wt. mg/100 cm. <sup>2</sup>	180 ± 7		189 ± 14		206 ± 21 S		212 ± 15 S		198 ± 23	
	Kidney wt. mg/100 cm. <sup>2</sup>	432 ± 27		486 ± 90		558 ± 57 S		592 ± 91 S		542 ± 59 S	
	Adrenal wt. mg/100 cm. <sup>2</sup>	8.0 ± 0.9		3.6 ± 1.0 S		7.5 ± 1.3		3.3 ± 0.4 S		7.3 ± 1.1	
	Plasma K	5.13 ± 0.39		5.63 ± 0.35		3.49 ± 0.53 S		3.67 ± 0.69 S		4.22 ± 0.59 S	
	Electrolytes Na	147.8 ± 7.9		145.4 ± 7.6		149.6 ± 9.5		154.8 ± 3.7		151.2 ± 8.8	
	mEq. Cl	129.8 ± 2.2		135.4 ± 2.4 S		122.8 ± 6.5		124.2 ± 6.3		126.0 ± 2.9	

Values significantly different from those of the control group are indicated by an S.

result would not appear to be an artifact referable to the failure of these animals to grow, since the increased heart weight is significant even in comparison with animals of group 2, receiving cortisone alone, whose heart weight was similar to that of the normal controls.

Cortisone alone caused a moderate increase in kidney size relative to surface area. This effect was apparently in the same direction as the increase in kidney weight caused by DCA, since in group 4, receiving DCA with cortisone, kidney weight was increased to a greater extent than in either group alone. Similarly, the marked reduction in size of the adrenals caused by cortisone was additive with the slight adrenal reduction caused by DCA.

Taken together, these results do not suggest that cortisone has any real antagonistic effect on the cardiovascular-renal effects of DCA. It would seem that cortisone here acted in the same direction as DCA on the cardiovascular-renal system, but less intensely, with the failure of blood pressure to increase along with heart and kidney weight being due largely to the debilitated state of the animals. The experiment will have to be repeated in mature animals, where the body weight is more stabilized before a definite statement on this can be made.

#### *Electrolytes*

The findings are presented in table 1. As usual, DCA caused a marked reduction in plasma potassium levels. Cortisone appeared to exert a weak effect in the opposite direction, but these results are not conclusive. On the other hand, cortisone appears to act in the same direction as DCA on plasma sodium, since only in the DCA-cortisone group was a possibly significant plasma sodium increase observed.

The plasma chloride levels suggest that cortisone retains potassium as KCl since like potassium, the chloride level is somewhat raised. In this effect, cortisone appears to act in a direction opposite to DCA which here seems to cause the loss of chloride as well as potassium.

While the effects of cortisone on electrolytes are thus at least partially opposite to those of DCA, it must be appreciated that the DCA effects are apparently more powerful, since although the DCA dose is small (about 300  $\mu$ g/day with all the pellets implanted) the DCA effect clearly overshadows that of cortisone when these steroids are given together.

#### *Histology*

**Kidney.** The kidneys of the cortisone-treated animals showed changes restricted to the glomeruli. As a uniform observation, it was noted that the capillary loops of the glomeruli were distended and in many instances formed large almost sinusoidal spaces. The intercapillary material was apparently increased in amount. No changes were observed in other parts of the vascular tree or nephron.

The kidneys of the DCA-treated animals showed the early changes usually noted upon administration of this steroid. These consist largely of minimal hyalinization of the tufts and, in some instances, of slight thickening of the arteriolar walls.

The animals receiving DCA with cortisone showed changes, chiefly in the glomeruli, which seemed to be the sum of the independent effects of each steroid.

These glomeruli were characterized by congestion, dilatation and confluence of the capillary loops, and early hyalinization of the tufts. Little change was seen in the remaining parts of the vascular tree or of the nephron.

**Liver.** Alterations in the liver were noted only in the cortisone-treated groups. In these animals, some atrophy of cells was noted with vacuolized spaces suggestive of a fatty infiltration. In view of the known effects of cortisone on carbohydrate metabolism, it is likely that these were deposits of glycogen. This change was also noted in the group receiving both DCA and cortisone.

**Adrenal.** The adrenal was markedly reduced in size in the cortisone-treated groups (table 1). In some of these animals, degenerative changes proceeding to frank necrosis were observed in the *zona reticularis*.

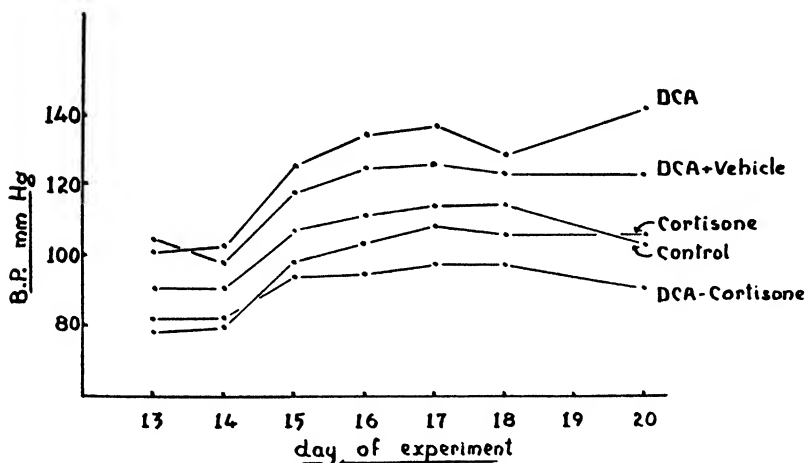


Fig. 1

**Intestine, spleen and pancreas.** These organs were not markedly altered. The spleen in some of the cortisone-treated animals showed a reduction in the size and amount of germinal lymph nodes with an absence of the usual differentiation between the central and peripheral parts of the germinating tissue. In addition, the pulp was relatively acellular.

The pancreas in the cortisone-treated groups appeared normal although the islet tissue seemed particularly abundant. This, of course, may be due to an overall reduction in the amount of acinar tissue.

**Heart.** No changes were observed in the myocardium in any group.

**Testis.** There was a tendency for suppression of spermatogenesis in the cortisone-treated groups although this was not uniform. In about half of these animals no sperm were found and in 2 the spermatogenesis had apparently not progressed beyond the stage of primary spermatocyte formation.

**Pituitary.** While no marked change was observed in the pituitary of these experimental animals there was a suggestion of a reduction in the number and size of

the eosinophils in the anterior lobe in about half of the cortisone-treated animals. This was noted in both groups receiving cortisone and would suggest that the growth inhibition observed in these animals was referable to a suppression of pituitary growth hormone.

#### DISCUSSION

While at first sight the data obtained in this experiment suggest that cortisone may antagonize the blood pressure raising effect of DCA, the simultaneous observation of an increased heart size in DCA-cortisone-treated animals indicates that the blood pressure effect may be largely due to the failure of these animals to gain weight normally. This receives further support from the fact that cortisone did not inhibit the renal effects of DCA. These findings are in partial agreement with those of Selye (9) and of Perera *et al.* (10) in man, but suggest that the depressor effect of cortisone depends largely upon other effects of its administration.

In this experiment, cortisone given alone caused an elevation in plasma potassium and chloride—effects opposite to DCA. In animals receiving DCA with the cortisone, however, the DCA effect was predominant.

In the dose used here, cortisone apparently exerts a damaging effect on the renal glomerulus characterized by an increased patency of the capillary loops and an increase in the amount of intercapillary material. This change occurs in the presence of DCA and is additive to the renal effects of the latter steroid.

In the liver, cortisone causes changes rather suggestive of a fatty infiltration. It is reasonable to assume that the infiltrating material may be glycogen.

The spleen shows a tendency to reduction in lymphoid tissue with a relative acellularity of the stroma as noted by Molomut *et al.* (11). The appearance of the pituitary indicates that there is probably a reduction in the number and quality of the eosinophils, which would account for the observed inhibition of growth in these animals, confirming the original observation of Wells and Kendall (12). This finding is also in agreement with the observation that the testis in cortisone-treated animals showed a partial suppression of spermatogenesis. Adrenal atrophy was also noted.

All of the morphological effects enumerated occurred not only in the group receiving cortisone alone but also in the group receiving DCA in addition to cortisone.

As an over-all conclusion it may be noted that as far as the data in this experiment are concerned, cortisone does not antagonize the cardiovascular-renal effects of DCA.

#### SUMMARY

Cortisone, alone and in combination with DCA, has been studied with regard to its cardiovascular-renal action and its effects on plasma electrolytes. In addition, routine histological sections of kidney, heart, adrenal, spleen, pancreas, intestine, testis and pituitary have been examined. The blood pressure rise which ordinarily follows the administration of DCA did not occur in the presence of cortisone. The heart and kidney weight increases caused by DCA were, however, not inhibited by cortisone. Thus, cortisone cannot be considered antagonistic to the cardiovascular-renal effects of DCA. Cortisone caused an elevation of plasma potassium and chloride,

effects opposite to those of DCA, but overshadowed by the latter when both steroids were given together. Cortisone caused renal glomerular lesions which were additive to those of DCA when both steroids were given together. The histological findings suggest that cortisone, among its other effects, tends to decrease the number of eosinophils in the anterior pituitary.

The cortisone acetate used in this work was obtained from Merck and Co., Ltd. through the courtesy of Dr. M. Darrach and with an allocation from the National Research Council of Canada. The DCA pellets were supplied by the Schering Corporation.

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# CYTOCHEMICAL STUDY OF THE ADRENAL CORTEX OF THE RAT UNDER SALT STRESSES

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THERE is much evidence to indicate that a relative independence exists between the zones of the adrenal cortex (1). Additional evidence to support this hypothesis has accumulated for the past few years. Deane and Greep (2) reported that the cortical atrophy following hypophysectomy is confined to the fascicular and reticular zones. In conditions eliciting an increased production of adrenocorticotrophin, cells of the inner zones of the cortex show signs of hyperactivity whereas the glomerular zone remains essentially normal in appearance (3). Essentially similar findings have been reported by Yoffey and Baxter (4) following injections of adrenocorticotrophin into rats. Hyperactivity of the glomerular zone has been observed in rats with decreased Na/K ratios (5, 6). The converse has been observed following an increased ratio (5).

Deane and Morse (7) reported cytochemical data in consonance with chemical findings which indicate very characteristic changes in the content of ascorbic acid in the adrenals of animals under various stresses. Injections of ACTH into rats led to depletion of ascorbic acid from the sinusoids of the inner zones; and the eventual depletion of cytochemically detectable ascorbic acid from the inner zones was observed following hypophysectomy. It seemed desirable to obtain information concerning changes in the cytological distribution of ascorbic acid in the glomerular zone of the rat under various stresses which affect the glomerular zone. In addition it seemed necessary to investigate the effect of alterations in dietary Na/K ratio on the adrenal cortex.

## MATERIALS AND METHODS

Male Sprague-Dawley rats were supplied *ad libitum* with Purina Laboratory Chow, and with tap water, 2.5 per cent KCl solution, or 2.5 per cent NaCl solution.

*Absolute Controls.* Weight measurements and histological preparations were made of the adrenals of 10 normal adult rats and 6 normal young rats.

*Sodium Chloride Flooding.* Nine young adult rats were placed in individual cages and each supplied with Chow and sodium chloride solution as the sole source of fluid. Three rats each were killed at the end of 9, 15 and 30 days, respectively.

*Potassium Chloride Flooding.* Nine rats were supplied with KCl solution as the sole source of fluid, and killed in groups as those above.

Four rats of similar weights and history as those of the above groups served as

additional control to both groups. These animals received tap water and Chow *ad libitum*.

**Sodium Chloride Injection.** Two rats, fed with Chow and tap water were given single injections of 2.5 per cent NaCl solution at the rate of 1.5 ml/100 gm. in the course of 15 minutes. Control animals received similar amounts of 0.85 per cent NaCl. These animals were killed at the end of one hour after injections.

**DCA Injections.** Nine rats on Chow and tap water were given single subcutaneous injections of a DCA suspension (2.5 mg/100 gm. body weight). Control animals received single subcutaneous injections of saline solution. Two DCA-treated animals and one control animal were killed one hour after injection, 3 DCA-treated and 3 control animals 3 hours after injection, and 4 DCA-treated and 3 control animals 24 hours after injection. No histological examinations were made of rats killed 3 hours after DCA treatment. Three rats were given daily subcutaneous injections of the DCA suspension (2.5 mg/100 gm/day), and killed at the end of 30 days. Two control rats received corresponding amounts of the saline solution at the same rate.

At the end of each experiment the adrenals were extirpated while the rats were under Evipal (hexobarbital soluble) anesthesia. The glands were cleaned of fat and, except in cases specifically mentioned, weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance. One gland from each animal was used for detection of ascorbic acid, and the other was fixed in 10 per cent neutralized formalin. This formalin-fixed tissue was used for detection of lipids, cholesterol and 'ketosteroids.'

**Histochemical Methods. Ascorbic acid detection.** Within one minute after extirpation, the gland was slit in the capsule and placed in a dark vial containing 5 per cent silver nitrate adjusted to pH 2 to 2.5. The vial was placed in an incubator maintained at 55° to 56° C. for  $\frac{3}{4}$  to one hour. At the end of this treatment the silver nitrate solution was poured off and distilled water added into the vial. After 10 to 15 minutes of washing, the water was poured off and an acidified sodium thiosulfate solution (5%) was introduced into the vial. The thiosulfate treatment, which lasted  $\frac{1}{2}$  to  $\frac{3}{4}$  hour, fixed the particles of reduced silver in the tissue. The tissue was then dehydrated in dioxan, embedded in paraffin (Fisher's Tissuemat), and sectioned (7  $\mu$  thick). Sections were cleared in xylene and mounted with Fisher's Permunt; some sections were stained with Delafield's hematoxylin eosin.

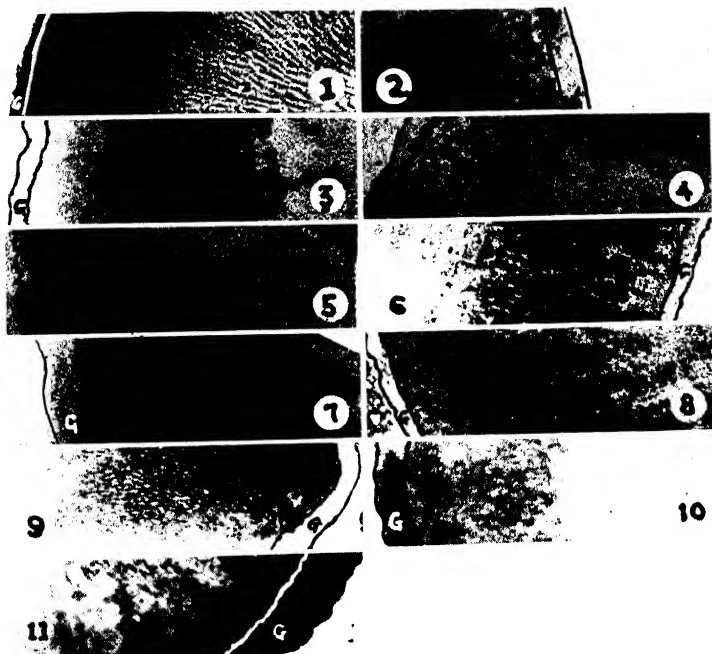
**Lipid detection.** Frozen sections (10-15  $\mu$ ) were cut of the formalin-fixed tissues. Separate sections of each gland were stained with Sudan IV; others were treated for the Plasmal, the Phenylhydrazine (8) and the Schultz (9) reactions. Some sections were treated with acetone to test solubility. All sections were mounted in glycerin jelly. Positive Plasmal and Phenylhydrazine reactions are considered to be indicative of carbonyl groups (8); the Schultz reaction is indicative of diols formed from cholesterol through mild oxidation procedures (10).

#### RESULTS AND OBSERVATIONS

**Adrenal Weight.** The data collected were not analyzed for statistical significance due to paucity of the samples. The adrenal weights of NaCl-treated and DCA-treated animals were less than those of normal control animals. NaCl-treated animals suffered profound weight loss. Animals under short-term treatments showed no marked changes in adrenal weights.



**Histological Observations.** The normal adrenal cortex. Sudanophilia is exhibited by the inner zones with a definite gradient of intensity weakest in the reticular zone, and greatest in the fascicular zone. A narrow sudanophobic zone, the 'transitional zone,' lies between the glomerular and fascicular zones. The glomerular zone is strongly sudanophilic (fig. 1).



Figs. 1-11. Photomicrographs of frozen sections of adrenals (10-15  $\mu$  thick). Demarcation lines are drawn in to indicate limits of the glomerular zone marked G. 1, Sudan stained, 2, Schultz-treated, adrenal of normal animal. Note gradient of intensity of lipid reactions, transitional zone partially covered by demarcation lines. 3, Sudan-stained adrenal of animal flooded with NaCl for 9 days. Note lipid depletion from glomerular zone. 4, Sudan-stained adrenal of animal flooded with KCl for 9 days. Note lipid depletion from glomerular zone. 5, Sudan-stained, 6, Schultz-treated, adrenal of animal flooded with NaCl for 15 days. Note depletion from glomerular zone. 7, Sudan-stained adrenal of animal flooded with KCl for 15 days. Note widening of glomerular zone associated with relative reaccumulation of lipids. 8, Sudan-stained, 9, Schultz-treated, adrenal of animal flooded with NaCl for one month. Note absence of lipids from the glomerular zone. Inner zones may be slightly widened. 10, Sudan-stained, 11, Schultz-treated, adrenal of animal flooded with KCl for one month. Note accumulation of lipids in the glomerular zone associated with widening of the zone.

**Cholesterol distribution.** A positive Schultz reaction is almost completely co-extensive with sudanophilia except in the reticular zone where this reaction is weakly positive (fig. 2).

**'Ketosteroid' distribution.** The Plasmal and the Phenylhydrazine positive areas are co-extensive with the Schultz-positive areas.

**Ascorbic acid distribution.** The Silver test indicates that a reducing substance is present in almost all cells of the cortex. The reducing property observed under these

conditions is mainly attributable to ascorbic acid (11). Granules of reduced silver are finely distributed in the glomerular zone, whereas somewhat coarser granules are present in the inner zones. On the basis of the distribution of ascorbic acid in cortical cells, two 'types' of cells are seen. In one type the ascorbic acid granules are aggregated peripherally close to the cell membrane. The author has observed in previous work in which the adrenals were fixed after an initial delay, that this distribution of cell 'types' does not occur. In such glands most of the ascorbic acid is aggregated perinuclearly. This perinuclear aggregation has been ascribed by Greep and Deane (12) to post-mortem changes in the tissues.

*Adrenals of experimental animals.* There was observed a considerable depletion of lipids, cholesterol and 'ketosteroids' from the glomerular zones of the 9-day-NaCl, and the 9-day-KCl-flooded animals. Ascorbic acid assumes a subcapsular clumping in this zone in sodium chloride-flooded animals. In the KCl-flooded animals, on the other hand, almost all glomerular zone cells exhibit a peripheral aggregation of ascorbic acid. In both the KCl and the NaCl-flooded animals the lipid and ascorbic acid pictures of the inner zones are essentially normal (figs. 3 and 4).

The glomerular zone of the 15-day-NaCl-flooded animal shows decreased lipid (fig. 5), cholesterol (fig. 6), and 'ketosteroid' tests associated with a subcapsular clumping of ascorbic acid. The adrenal of the 15-day-KCl-flooded animal shows a widened glomerular zone with sudanophilia of normal intensity (fig. 7). Most of the glomerular zone cells exhibit peripheral aggregation of ascorbic acid. The inner zones are essentially normal in this animal.

After 30 days of sodium chloride flooding the glomerular zone is decreased in width and shows a complete disappearance of lipids (fig. 8), of cholesterol (fig. 9), and of 'ketosteroids.' Large clumps of ascorbic acid occur at the periphery of this atrophied zone. The inner zones appear normal in staining properties and may even be somewhat wider than normal.

The glomerular zone of the 30-day-KCl-flooded animal is considerably widened and it apparently envelops the transitional zone. It exhibits a great increase in sudanophilia (fig. 10) and the Schultz reaction is enhanced (fig. 11). Most of the cells appear larger and exhibit subnormal amounts of peripherally aggregated ascorbic acid granules. The appearance of the 30-day-DCA-treated animal's adrenal is identical with that of the animal treated with NaCl for a similar period except for a slight decrease of lipids from the reticular zone which is observed in DCA-treated animals.

Signs of the alarm reaction, lipid, cholesterol and 'ketosteroid' depletion from the inner zones, associated with decrease and peripheral aggregation of ascorbic acid, are observed in the adrenals of animals receiving injections of sodium chloride. The control animals reacted the same way. This finding apparently indicates that the nonspecific stress of infusion of fluid into the body may be responsible for these changes.

There is no major change in the adrenal following a single injection of DCA. Three to 24 hours after injection there is a slight clumping of ascorbic acid and of lipids in the glomerular zone. No change was observed in the inner zones of these animals receiving single injections of DCA.

## DISCUSSION

One of the more basic experiments designed to prove whether an endocrine organ produces a certain factor is the demonstration of disuse atrophy of that organ following a sufficient exogenous supply of the factor. The DCA study was an experiment of this nature; DCA is a synthetic compound that closely imitates the action of the natural hormone. Signs of structural and of functional atrophy of the glomerular zone of the cortex were observed after the rat was given an exogenous supply of DCA for one month. This finding is in consonance with the findings reported by Deane, Shaw and Greep (5), and by Knowlton *et al.* (6). The clumping of steroids and of ascorbic acid in the glomerular zone of the animal receiving one injection of DCA may be interpreted as a preliminary step to disuse. In this specific case, the clumping indicates 'storage' of the hormone or of the precursors to the hormone.

Since the major function of desoxycorticosterone-like hormones seems to be maintenance of the Na/K ratio, then it is expected that extrinsic influences tending to maintain this ratio will induce signs of disuse of the glomerular zone should the maintenance prolong for some adequate time. This is precisely the finding from the experiment with sodium flooding for one month. The findings from this type of maintenance for shorter periods indicate the progressive nature of this atrophy of disuse. The changes undergone by ascorbic acid in the cells during the various stages are well worth noting. The ascorbic acid clumps under the capsule of the gland in such a manner as to suggest that it becomes 'inert' insofar as its participation in the secretion of cortical hormones is concerned.

Potassium flooding, a factor which tends to depress the Na/K ratio in the body, induces the opposite change in the glomerular zone. In contrast to the sodium-induced progressive atrophy of disuse, a progressive hypertrophy and hyperactivity are elicited by potassium flooding. Following 9 days of potassium flooding signs of the alarm reaction, lipid depletion associated with peripheral aggregation of ascorbic acid, are observed in the glomerular zone. Following 15 days and 30 days of potassium flooding, signs of the resistance phase of the General Adaptation Syndrome are observed. This phase is characterized by reaccumulation of lipids, enlargement of the cells, and peripheral aggregation of ascorbic acid. These signs are restricted to the glomerular zone.

It is possible that the stimulus-response circuit under which the glomerular zone operates is essentially different from the circuit under which the inner zone operates. It has been observed that single injections of hypertonic and of isotonic salt solutions do not elicit any reaction in the glomerular zone. These treatments do elicit signs of the alarm reaction in the inner zones. This response of the inner zones may be entirely nonspecific.

No attempt is made in this paper to discuss the role of ascorbic acid in the adrenal cortex, but the changes in steroid distribution associated with the ascorbic acid shifts are worthy of further study. Strong indirect evidence exists that cholesterol is eventually converted into cortical hormones (13). Physiological conditions causing a sudden increased demand for cortical hormones lead to a decreased concentration of cholesterol in the adrenal gland; the ascorbic acid content of the cortex parallels

this pattern (13). Zwemer and his colleagues have suggested the association of ascorbic acid with the formation and secretion of cortical hormones. These workers have also reported the isolation of an ascorbic acid steroid factor from the adrenal gland which is high in corticosteroid activity (14). The ascorbic acid shift in adrenals observed in these studies also suggests an intimate involvement of ascorbic acid in the metabolism of the adrenal cortex, and possibly to the secretion of hormones by the adrenal cortex.

#### SUMMARY

Histological and histochemical changes in the adrenal cortex of the rat treated with NaCl, KCl, and DCA were observed. Signs of progressive functional atrophy were observed in the glomerular zone of the rat flooded with either NaCl or with DCA. Progressive functional hypertrophy was observed in the glomerular zone of the rat treated with KCl. It is suggested that these changes were induced by changes in the dietary Na/K ratio. This explanation is in complete consonance with the hypothesis that the salt water hormone is secreted by the glomerular zone, and that the major function of the hormone is the maintenance of the Na/K ratio. The ascorbic acid shift observed in this study strongly suggests that ascorbic acid is actively participating in the metabolic and secretory activity of the adrenal cortex.

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# SITES AND NATURE OF PROTEIN ANABOLISM STIMULATED BY TESTOSTERONE PROPIONATE IN THE RAT<sup>1,2</sup>

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THE protein anabolic effect of the androgens is now well known (1). It has been demonstrated, recently, however, that in the castrated (2), hypophysectomized (3) or adrenalectomized (4) rat, the ability of androgens to cause nitrogen retention disappears on extension of injections and at the same time a loss in the gained body weight occurs. The early gain in body weight could be accounted for by the conversion of the retained nitrogen to tissue but the loss in body weight with continued injections was not accompanied by a concomitant loss in nitrogen. The discrepancy between the observed and calculated body weight on extension of the androgen injections became greater with higher doses of androgens (2). In order to explain these phenomena the analyses of the various tissues of the rat under different conditions of treatment with androgen were undertaken.

## METHODS

*Procedure.* Rats were kept in individual cages in an air-conditioned room maintained at 25.5° to 26.6° C. Castration was performed under ether anesthesia.

The rats were fed and weighed at the same time each day. The diet consisted of casein 16.7, sucrose 61, hydrogenated vegetable oil 7.4, cellul flour 1.8, Wesson's salts 3.7 and brewers' yeast (Fleischmann's 2019) 9.3. In addition a daily supplement of one drop of cod liver oil and one drop of a 34 per cent tocopherol concentrate of wheat germ oil diluted tenfold with Wesson oil was given.

At the end of the experiments the rats were anesthetized with dial-urethane<sup>4</sup>, bled by cutting the neck, the organs removed and weighed on a Roller-Smith torsion balance and prepared for analyses. Fecal material in the gastro-intestinal tract was weighed and discarded.

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<sup>1</sup> This investigation was initiated under grants from Ciba Pharmaceutical Products Inc. and completed under a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

<sup>2</sup> Parts of these data have been reported in a review Symposium on Steroid Hormones, Madison, Wisconsin 1950, and in the Josiah Macy Jr. Foundation Conferences on The Metabolic Aspects of Convalescence, thirteenth meeting 49 (1946).

<sup>3</sup> Some of these data have been taken from a thesis submitted in January 1948 by M. N. Bartlett to the Graduate School of the University of Rochester in partial fulfillment of the M.S. degree.

<sup>4</sup> The dial-urethane and the testosterone propionate (Perandren) used in these studies were generously supplied by Ciba Pharmaceutical Products Inc.

**Analyses. Water.** The carcasses of the rats fed *ad libitum* were ground in a Hobart meat grinder and those of the rest of the experiments were dismembered into several pieces, placed in a 120-mm. crystallizing dish and dried at 90° to 100° C. to constant weight in an electric oven to which a water aspirator was attached. The various organs were placed as such either in beakers or on aluminum foil and dried. Part of the liver and kidney was removed at autopsy for other analyses.

**Fat and nitrogen.** The dried tissues were dissolved in equal parts of 50 per cent potassium hydroxide and redistilled ethyl alcohol. Aliquots were analyzed for fat and nitrogen as previously described (5).

**Non protein and protein nitrogen:** The protein of plasma or tissue was precipitated by 10 per cent trichloroacetic acid. The tissue was quickly cut into small pieces, added to the 10 per cent trichloroacetic acid and homogenized in an apparatus of the Potter-

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON BODY WEIGHT AND CARCASS OF THE CASTRATED RAT<sup>1</sup>

TREATMENT	RAT NO.	BODY WEIGHT		CARCASS			
		Initial	Change	Wgt.	H <sub>2</sub> O	Prot.	Fat
		gm.	gm.	gm.	%	%	%
Controls. . . . .	14	298	-1	263.1	61.1	19.9	13.2
T.P. 0.125 mg/day, 21 days. . . . .	10	298	+15	275.7	61.1	19.7	13.0
T.P. 1.0 mg/day, 21 days. . . . .	10	294	+15	271.2	61.6	19.5	12.9
T.P. 1.0 mg/day, 46 days. . . . .	9	292	+9 <sup>2</sup>	260.3	62.0	20.1	11.9

<sup>1</sup> Detailed data for this table have been deposited with the American Documentation Institute, 1719 N Street, N. W., Washington 6, D. C. For copies of this table order Document 2914 directly from the Institute, remitting \$0.50 for microfilm (images 1 inch high on 35-mm. film) or \$0.50 for photocopies (6 x 8 inches).

<sup>2</sup> This group of rats attained a maximum increase in body weight of 17 gm. at 12 days which decreased from the 21st day to the end of the experiment.

Elvehjem type. The nitrogen content of the soluble (non protein) and insoluble (protein) portions were determined by the micro-Kjeldahl procedure.

**Amino acids.** The total amino acids were determined by the Albanese-Irby chemical method (6) and the individual amino acids by paper chromatography (7). The individual amino acids were compared (7) on the basis of area and intensity of color developed by ninhydrin and graded from 1 to 10 with the exception of proline which was graded from 1 to 3 since it produces a yellow color instead of the purple of the other amino acids.

## RESULTS

**Castrated Rats on Constant Food Intake.** The rats were of the Carworth-Sherman strain and weighed 270 to 300 gm. on purchase. They were placed in individual cages, castrated and fed 12 gm/day of the prepared diet. The food intake was gradually reduced to a quantity which maintained constant body weight. The rats were then

divided by weight into the various groups for injections. The first of this series was begun 5 months after castration.

**Body weight.** The expected (2) increases in body weight (table 1) were observed. The two groups treated for 21 days had attained their maximum weight increase at 14 and 15 days and had maintained it to autopsy. The rats treated for 46 days reached a maximum increase in body weight of 17 gm. at 12 days, maintained this maximum until the 21st day then gradually lost weight so that at 46 days their body weights represented an increase of only 9 gm.

**Composition of tissues.** The percentage composition (tables 1, 2) of the carcass and the various organs showed no remarkable changes except the fat content of the

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON WEIGHT AND COMPOSITION OF ORGANS OF THE CASTRATED RAT

	WT.	H <sub>2</sub> O	PROT.	FAT	WT.	H <sub>2</sub> O	PROT.	FAT
	gm.	%	%	%	gm.	%	%	%
		<i>Liver</i>				<i>Kidney</i>		
Controls.....	7.88	70.8	20.7	2.76	2.01	75.8	17.5	2.62
T.P. 0.125 mg/day, 21 days.....	8.92	70.2	20.6	2.39	2.09	74.3	18.6	2.77
T.P. 1.0 mg/day, 21 days.....	8.41	70.3	21.1	3.06	2.21	74.3	18.0	2.74
T.P. 1.0 mg/day, 46 days.....	8.62	70.6	19.8 Rest %	3.16	2.37	74.4	18.7	3.41
		<i>Gut<sup>1</sup></i>				<i>Sem. Ves. &amp; Pros.</i>		
Controls.....	18.4	66.4	11.2	15.4	0.24	76.9	15.1	4.24
T.P. 0.125 mg/day, 21 days.....	18.9	65.0	10.9	17.6	2.31	75.2	14.9	5.23
T.P. 1.0 mg/day, 21 days.....	18.7	63.1	10.1	18.2	4.51	74.9	17.0	3.45
T.P. 1.0 mg/day, 46 days.....	18.2	64.6	11.8	17.9	5.71	77.1	16.1	2.53

<sup>1</sup> Fecal contents of the cecum and rectum were discarded. They weighed 5.0, 4.3, 4.9 and 4.9 gm. for the respective groups.

seminal vesicles and prostates which decreased with prolonged stimulation by the androgen.

The composition of the changes (table 3) in body weight was obtained by subtraction of the control values from those of the injected animals and with correction for the small differences in initial body weight. The main site of increase in weight was the carcass but as the dosage and duration of treatment was increased there was a shift from the carcass to the accessory sex organs and to a smaller degree the kidney so that the rats treated for 46 days had increased their seminal vesicles and prostates by 5.48 gm. and the carcass by only 2.44 gm. These changes were accompanied by proportionate changes in protein and water and in the rats treated for 46 days a loss in carcass fat. The liver also was a site of synthesis of new tissue which was primarily protein but no correlation with duration of treatment was evi-

dent. The proportion of water to protein retained in all of the tissues is that observed in young growing animals (8).

*Castrated 'Fat' Rats on Constant Food Intake.* These rats also of the Carworth-Sherman strain were castrated at a much younger age, 180- to 200-gm. body weight, allowed to eat *ad libitum* for 3 months until they were about 300 gm. in body weight, then their food was reduced until body weight and nitrogen equilibrium was attained. The injections of testosterone propionate at one mg/day for 13 days were not begun until 7 and 11 months after castration. The rats were autopsied on the 14th day after injection.

TABLE 3. CHANGES IN COMPOSITION OF CASTRATED LEAN RATS PRODUCED BY TESTOSTERONE PROPIONATE<sup>1</sup>

	CARCASS	SEM. VES. AND PROS.	LIVER	KIDNEY	GUT	TOTAL
	gm.	gm.	gm.	gm.	gm.	gm.
<i>0.125 mg/day; 21 Days</i>						
Weight.....	12.60	2.08	1.04	0.08	0.53	16.33
Water.....	7.33	1.72	0.68	0.03	0.13	9.89
Protein.....	1.89	0.31	0.20	0.04	-0.01	2.43
Fat.....	1.09	0.12	0.00	0.01	0.48	1.70
<i>1.0 mg/day; 21 Days</i>						
Weight.....	11.60	4.28	0.53	0.20	0.48	17.09
Water.....	8.08	3.36	0.33	0.12	-0.30	11.89
Protein.....	1.18	0.73	0.15	0.04	-0.16	1.94
Fat.....	0.99	0.14	0.04	0.01	0.62	1.80
<i>1.0 mg/day; 46 Days</i>						
Weight.....	2.44	5.48	0.74	0.36	0.16	9.20 <sup>2</sup>
Water.....	3.48	4.39	0.50	0.24	-0.24	8.37
Protein.....	0.86	0.88	0.08	0.09	0.11	2.02
Fat.....	-2.96	0.14	0.06	0.03	0.44	-2.27

<sup>1</sup> Values represent the difference between the injected and control rats.

<sup>2</sup> This group of rats attained a maximum increase in body weight of 17 gm. at 12 days.

*Body weight and urinary nitrogen.* The testosterone propionate increased the body weight by 13 gm. and caused a total nitrogen retention of 554 gm. and an average maximum daily nitrogen retention of 52 mg. (table 4) (cf. 2). Thus, the increase in body weight as calculated by multiplying the nitrogen retained by the factor 29.2 (9) is in good agreement with the observed increase (tables 4, 6).

*Composition of tissues.* Apparently castration at the younger age plus the longer period of castration prior to injection of the androgen resulted in a greater deposition of body fat which was markedly decreased by the androgen (tables 4, 6). The other tissues (table 5) show the same changes as seen in the previous experiments (tables 1-3).

The carcass is the main site of protein synthesis (table 6) which is accompanied



by the deposition of an amount of water characteristic of new tissue (8). The other organs also are sites of synthesis. The amount of total new protein is in good agreement with that determined by multiplying the retained urinary nitrogen by 6.25. It is noteworthy that the total protein synthesis in this experiment is greater than that in any of the experiments with the 'lean' rats. This may be due to experimental variation; on the other hand, the difference may be due to the nutritive state of the animal, the availability of easily accessible fat for caloric purposes permitting a greater protein-anabolic effect of the androgen.

TABLE 4. EFFECT OF ONE MG/DAY OF TESTOSTERONE PROPIONATE FOR 12 DAYS ON BODY WEIGHT AND NITROGEN EXCRETION OF CASTRATED RATS

	NO.	URINE NITROGEN			BODY WEIGHT		
		Preinject.	Retained		Initial	Change	Calc'd <sup>1</sup>
			Max/day	Total			
		mg/day	mg.	mg.			
Controls.....	9	219	4	39	311	+1	+1
T.P. at 1.0 mg/day, 13 days.....	13	216	52	554	302	+13	+16

<sup>1</sup> Nitrogen retained multiplied by the factor 29.2.

TABLE 5. EFFECT OF ONE MG/DAY OF TESTOSTERONE PROPIONATE FOR 13 DAYS ON COMPOSITION OF THE CARCASS AND ORGANS OF CASTRATED RATS

	WT.	H <sub>2</sub> O	PROT.	FAT	WT.	H <sub>2</sub> O	PROT.	FAT
	gm.	%	%	%	gm.	%	%	%
	<i>Sem. Ves. &amp; Pros.</i>				<i>Liver</i>			
Control.....	0.10	80.0	14.0	4.50	8.07	68.6	21.1	2.52
T.P.....	2.34	80.4	16.8	2.05	8.84	68.6	20.6	2.17
	<i>Carcass and Gut<sup>1</sup></i>				<i>Kidney</i>			
Control.....	291.6	55.4	18.3	20.8	1.84	75.3	18.6	1.35
T.P.....	292.0	59.8	19.0	15.5	2.23	74.1	18.9	1.38

<sup>1</sup> Fecal contents of the cecum and rectum were discarded. They weighed 10.2 gm. in the control and 9.5 gm. in the treated group.

*Castrated Rats Fed ad Libitum.* The rats were from our colony of original Wistar stock. They were castrated approximately 30 days prior to injections (figs. 1-3), placed in individual cages and body weight and food intake recorded daily.

*Food intake.* The rats injected with testosterone propionate ate more food than the control rats (table 7, fig. 1). The increased intake, however, did not become apparent until about 60 days after the beginning of the injections when the control rats began to eat gradually smaller and the experimental rats larger amounts of food. At the end of the experiment, 125 days, the injected rats were eating approximately 20 per cent more food than the controls.

*Body weight.* The androgen produced a sharp increase followed by a decrease in the rate of gain in body weight (figs. 1-3). The initial body weight of the animals

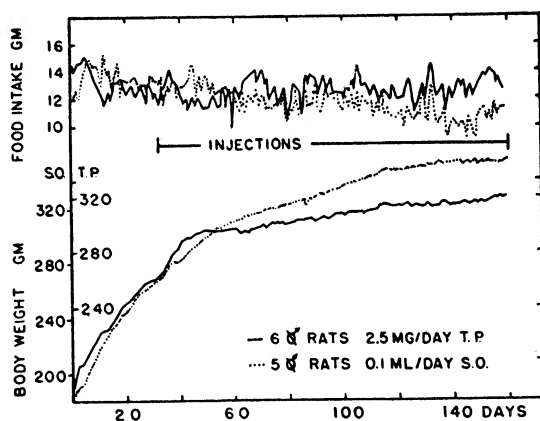
affected the degree and duration of these effects. The larger rats (figs. 1, 3) attained a maximum increase in body weight in 8 to 10 days after which the rate decreased to less than that of the control rats in 20 days while the smaller rats (fig. 2) attained these effects in one half the time.

TABLE 6. CHANGES IN COMPOSITION OF CASTRATED 'FAT' RATS PRODUCED BY TESTOSTERONE PROPIONATE AT ONE MG/DAY FOR 13 DAYS

	WEIGHT gm.	H <sub>2</sub> O %	PROTEIN gm.	FAT gm.	REST gm.
Carcass and gut.....	9.9	18.70	3.82	-13.49	0.87
Sem. ves., pros.....	2.24	1.80	0.38	.04	0.02
Liver.....	1.03	0.70	0.17	-.01	0.17
Kidney.....	.45	0.32	0.09	.01	0.03
Total.....	13.62	21.52	4.46	-13.45	1.09

The depressing effect of testosterone propionate on body weight never was sufficient to cause a loss in body weight even after the injections were continued for 125 days (fig. 1). Indeed there was a small but constant increase until the end of the experiment at which time the control rats, however, had gained 24 grams more in body weight in spite of the fact that they had consumed less food than the injected rats (fig. 1, table 7).

Fig. 1. EFFECT OF TESTOSTERONE PROPIONATE ON THE FOOD INTAKE AND BODY WEIGHT OF CASTRATED RATS. Castration performed at zero day.



*Hair and skin.* The hair of the androgen-treated rats became coarse, sparse and rusty in color within a few weeks after the beginning of the experiment. The skin also became rusty in color especially on the back.

*Organ weights.* The injection of testosterone propionate for 8 and 16 days increased the weight of the seminal vesicles and prostates to about one half and for 125 days to more than twice the weight found in normal rats (table 11). The androgen produced a marked increase in kidney size only after injection for 125 days. The shorter periods of treatment produced very small increases. Testosterone propionate produced a small increase in the weight of the liver but extension of the injections did not enhance the effect.

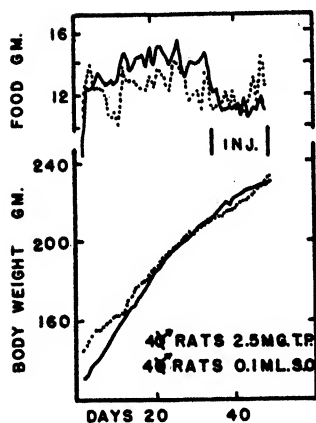


FIG. 2

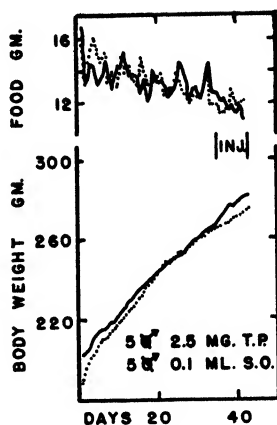


FIG. 3

Fig. 2. (left) EFFECT OF TESTOSTERONE propionate on the food intake and body weight of 'young' castrated rats. Castration performed at zero day.

Fig. 3. (right) EFFECT OF TESTOSTERONE propionate on the food intake and body weight of castrated rats. Castration performed at zero day.

TABLE 7. EFFECT OF TESTOSTERONE PROPIONATE (2.5 MG/DAY) ON FOOD CONSUMPTION, AND BODY AND ORGAN WEIGHTS OF CASTRATED MALE RATS

TREATMENT	RATS	FOOD CONSUMED		BODY WT.		SEM. VES. AND PROS.	KIDNEY	LIVER
		Total	Daily	Begin	Gain			
		gm.	gm.	gm.	gm.			
8-Day Experiment								
Castrated.....	5	107.0	11.90	266	10	0.15	1.80	7.8
Castrated and T.P.....	5	107.3	11.92	268	15	1.80	2.21	9.0
Diff.....		+0.3	+0.02		+5	+1.65	+0.41	+1.2
16-Day Experiment								
Castrated.....	4	171.6	11.44	212	21	0.08	1.66	6.7
Castrated and T.P.....	4	177.6	11.84	213	17	1.90	1.94	7.0
Diff.....		+6.0	+0.40		-4	+1.82	+0.28	+0.3
125-Day Experiment								
Castrated.....	5	1445.3	11.56	277	63	0.08	1.90	9.3
Castrated and T.P.....	6	1560.0	12.48	268	39	7.60	3.20	10.3
Diff.....		+114.7	+0.92		-24	+7.52	+1.30	+1.0

*Composition of the tissues.* The carcasses of the treated rats contained a higher percentage of water than those of the controls (table 8). This effect became very marked only on prolonged treatment, 125 days, and was due to a greater deposition of fat by the control rats. The water value of the fat-free carcass was the same for

both groups in all of the experiments. The percentage nitrogen of the carcasses of the rats treated for 125 days was somewhat greater than that of the control rats. The difference again was due to the greater fat content of the control rats. The percentage nitrogen in the fat-free carcass was the same in both groups of animals. There was, however, an apparent age effect. The carcasses of the older animals, 125 days of injection, had a smaller percentage of nitrogen than those of the younger rats.

The percentage of water and nitrogen in the liver and kidney (table 9) was not remarkably changed by testosterone propionate. These constituents of the seminal

TABLE 8. EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON COMPOSITION OF THE CARCASSES OF CASTRATED RATS

TREATMENT	CARCASS			FAT-FREE CARCASS	
	Water	Nitrogen	Fat	Water	Nitrogen
	%	%	%	%	%
<i>8-Day Experiment</i>					
Castrated.....	62.0	2.98	14.3	72.1	3.46
Castrated and T.P.....	63.8	3.07	12.0	72.1	3.53
Diff.....	+1.8	+0.09	-2.3	0.0	+0.07
<i>16-Day Experiment</i>					
Castrated.....	62.2	3.06	12.9	71.1	3.45
Castrated and T.P.....	63.1	3.01	11.7	71.5	3.39
Diff.....	+0.9	-0.05	-1.2	+0.4	-0.06
<i>125-Day Experiment</i>					
Castrated.....	54.9	2.48	23.7	71.5	3.19
Castrated and T.P.....	60.7	2.67	15.7	72.4	3.16
Diff.....	+5.8	+0.19	-8.0	+0.9	-0.03

vesicles and prostates were the same for the different periods of injection. The accessory sex organs of the control rats were not analyzed.

*Sites and nature of growth.* Prolonged injection, 125 days, of testosterone propionate decreased the gain in weight of the carcass due to a storage of less water, protein and especially fat (table 10). The organs of the injected rats gained more weight due to a greater content of water and protein than those of the controls. The increase in weight of the organs was only about 30 per cent of the loss in carcass weight. On the other hand, the increased storages of water and nitrogen by the organs were more than those lost by the carcass. The seminal vesicles and prostates due to their relatively tremendous increase in size contributed the greater portion, 76 to 79 per cent, of these gains.

*Normal Rats Fed ad Libitum.* *Body weight and food intake.* The rats of our colony were deprived of food for 7 days then allowed to eat *ad libitum* and injections were

begun. The effect of the androgen on the body weight was very similar to that observed in the castrated rat (fig. 4). The food intake, however, was only slightly increased (fig. 4, table 11).

*Organ weight.* The weight of the kidney was only slightly increased and that of the liver was not changed. The seminal vesicles and prostates, on the other hand, were greatly enlarged.

*Composition of the tissues.* The percentage composition of the tissues (table 12) showed no remarkable changes as a result of androgen injection. Therefore, since the body weight of the treated rats was less than that of the controls, the prolonged treatment with androgen produced an overall decrease in all of the tissue constituents.

TABLE 9. EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON COMPOSITION OF ORGANS OF CASTRATED RATS<sup>1</sup>

TREATMENT	LIVER			KIDNEY		SEM. VES. & PROS.	
	Water	Nitrogen	Fat	Water	Nitrogen	Water	Nitrogen
	%	%	%	%	%	%	%
<i>8-Day Experiment</i>							
Castrated.....	74.8	3.54	6.16	76.8	3.13		
Castrated and T.P.....	74.1	3.36	4.23	76.1	3.07		3.01
	-0.7	-0.18	-1.93	-0.7	-0.06		
<i>16-Day Experiment</i>							
Castrated.....	66.6	3.26	3.94	74.4	3.14		
Castrated and T.P.....	68.4	3.18	4.51	75.7	3.21	73.3	2.84
	+1.8	-0.08	+0.57	+1.3	+0.07		
<i>125-Day Experiment</i>							
Castrated.....	73.0	3.03	6.32				
Castrated and T.P.....	71.2	3.04	4.47			72.6	2.99
	-1.8	+0.01	-1.85			.	

<sup>1</sup> Omitted values were not determined.

Furthermore, since the liver was not changed, the kidneys slightly increased and the seminal vesicles and prostates greatly increased, the carcass was the site of loss of these constituents.

It is of interest that the percentage of fat in the carcass of the normal rats is nearly identical to that of the castrated rats treated with androgen for 125 days (table 8).

*Urine, Plasma and Tissue Amino Acids.* These studies were performed on the 'fat' castrated rats on constant food intake (cf. tables 4-6).

*Excretion of amino acids.* The androgen caused a suggestive slight decrease in the total amino acids of the urine from the second to the eighth day. Paper chromatography both by the single (table 13) and the two dimensional (table 14) technics, however, showed no remarkable changes. The greater number of amino acids detec-

ted in the unhydrolyzed urines (table 14) was due to the use of larger aliquots. The quantities, however, were not proportionately greater due to the splitting of peptides by the hydrolysis (10).

TABLE 10. EFFECT OF 125 DAYS OF INJECTIONS OF TESTOSTERONE PROPIONATE (T.P.) ON THE COMPOSITION OF GAIN IN BODY WEIGHT OF CASTRATED RATS FED AD LIBITUM<sup>1,2</sup>

	CARCASS	LIVER	KIDNEY	SEM. VES. AND PROS.	TOTAL
	gm.	gm.	gm.	gm.	gm.
Weight.....	-34.30	+1.00	+1.30	+7.52	-24.48
Water.....	-1.00	+0.54	+0.99	+5.41	+5.94
Protein.....	-2.92	+0.33	+0.67	+3.65	+1.73
Fat.....	-30.6	-0.13	+0.05 <sup>3</sup>	+0.19 <sup>3</sup>	-30.49

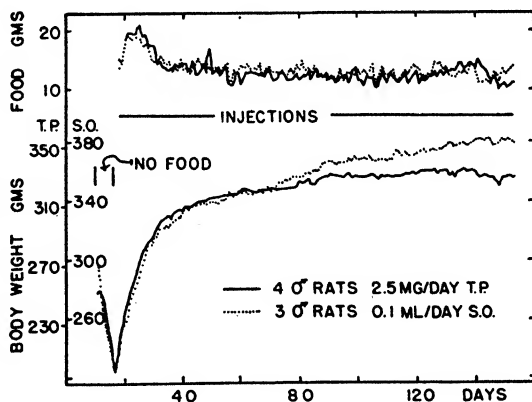
<sup>1</sup> Detailed data for this table have been deposited with the American Documentation Institute, 1719 N Street, N. W., Washington 6, D. C. For copies of this table order Document 2914 directly from the Institute, remitting \$0.50 for microfilm (images 1 inch high on 35-mm. film) or \$0.50 for photo copies (6 x 8 inches).

<sup>2</sup> Values are the differences between the changes in the androgen treated and control rats during the 125 days.

<sup>3</sup> These values were not determined directly but by assuming that the percentage composition in these organs was the same as those in table 2.

*Plasma nitrogen constituents.* The total non-protein and total amino acid nitrogen of the plasma taken at autopsy were identical for both groups of rats. The amino acids were not partitioned by paper chromatography.

Fig. 4. EFFECT OF TESTOSTERONE propionate on the food intake and body weight of normal rats after a 7-day fast.



*Tissue non-protein nitrogen and amino acids.* The tissues showed no percentage change in the non-protein nitrogen values as a result of androgen injections. The absolute values changed in proportion to the changes in the tissue mass. Furthermore, the individual amino acids (table 15) showed no remarkable changes either as whole tissue or as trichloroacetic acid precipitable protein except for the kidney in which

taurine and hydroxylysine were detected in the whole tissue. The lack of tryptophan is due to the method of hydrolysis.

#### DISCUSSION

The sites of protein synthesis were as expected from previous studies on organ weight in rats (cf. 1), mice (cf. 1, 11), hamsters (12) and guinea pigs (13); the skeletal muscle of guinea pigs (13) and also the analyses of the carcass and organs of the mouse after treatment with a pellet of testosterone propionate (5).

TABLE 11. EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON BODY WEIGHT, FOOD CONSUMPTION AND ORGAN WEIGHTS OF NORMAL MALE RATS<sup>1</sup>

	CONTROL <sup>2</sup> (3)	T.P. <sup>3</sup> (4)	DIFFERENCE	
	gm.	gm.	gm.	%
Body weight, beginning.....	295	277		
Body weight, end of 7-day fast.....	224	198		
Body weight loss.....	-71	-79	-8	+11.3
Body weight at autopsy.....	379	330		
Body weight gain.....	+155	+132	-23	-14.8
Food consumed/rat, total.....	1784.5	1831.7	+47.2	+2.6
Food consumed/rat, daily.....	13.12	13.47	+0.35	+2.6
Seminal vesicles & prostates.....	3.10	7.70	+4.60	+148.5
Kidney.....	2.37	2.62	+0.25	+10.5
Kidney/100 gm. body weight.....	.625	.794	+1.69	+27.1
Liver.....	8.20	7.0	-1.20	-14.6
Liver/100 gm. body weight.....	2.16	2.12	-0.04	-1.9

<sup>1</sup> Rats placed in individual cages, fasted for 7 days then allowed to eat *ad libitum*. The amount eaten was determined for each day.

<sup>2</sup> Injected subcutaneously with 0.1 ml/day of sesame oil for 136 days, i.e. from end of fast to autopsy. Figure in parenthesis represents number of rats.

<sup>3</sup> Injected subcutaneously with 2.5 mg/day of testosterone propionate (T.P.) (perandren) for 136 days, i.e. from end of fast to autopsy. Figure in parenthesis represents number of rats.

The appearance of a very marked effect on kidney weight by the androgen only after prolonged treatment provides an explanation for some of the early discrepancies with respect to the effect of androgens on this organ of the rat.

The 'wearing off effect' of the androgen on nitrogen retention with a concomitant decrease in body weight can now be attributed to a loss of body fat accompanied by a shift of carcass protein to the continued synthesis of new tissue at other sites especially the accessory sex organs and to a smaller degree the kidneys. It is interesting that the liver shows only an initial increase in weight which is not continued by further injections of the androgens.

The priority for growth endowed upon the accessory sex organs and the kidneys by the androgen is reminiscent of the priority endowed in a tumor but in this instance the stimulus for this property is not known. Androgens, however, have been found to be beneficial in certain types of tumor, e.g. mammary cancer. Thus, it may be construed that the androgen has placed this tumor low on the priority list and

diverted building materials to other tissues. On the other hand, the exacerbation of a tumor, e.g. carcinoma of the prostate, by androgens would be due to the fact that the tumor tissue is such that it is placed high on the priority list for building materials by the androgens. Thus, the effect of the androgen would be determined by the

TABLE 12. EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON COMPOSITION OF CARCASS AND ORGANS OF NORMAL ADULT RATS

	WATER			NITROGEN			FAT		
	Control	T.P.	Diff.	Control	T.P.	Diff.	Control	T.P.	Diff.
	%	%		%	%		%	%	
Carcass .....	61.1	60.8	-0.3	2.86	2.80	-0.06	14.20	14.90	+0.70
Liver .....	70.7	70.5	-0.2	3.67	3.74	+0.07	4.32	4.43	+0.11
Kidney .....	76.4	77.6	+0.8	3.03	2.94	-0.09			
Sem. ves. & prostates	69.8	73.0	+3.2	2.65	2.70	+0.05			
<i>Fat-Free Basis</i>									
Carcass .....	71.2	71.4	+0.2	3.33	3.30	-0.03			

TABLE 13. SINGLE STRIP CHROMATOGRAMS OF UNHYDROLYZED URINE<sup>1</sup>

	PREINJECTION PERIODS			INJECTION PERIODS				
	1	2	3	1	2	3	4	
Period.....	50	50	50	50	100	200	200	200
Urine aliquot $\mu$ l. ....								
Aspartic acid								
Exptl.....						T	T	T
Control.....						T	T	T
Glutamic acid								
Exptl.....	2.5	1	1	1.5	1.5	2	1.5	2.5
Control.....	1.5	1	1	2	2.5	2.5	1	2
Glycine								
Taurine								
Exptl.....	2.5	1.5	1.5	1	1.5	1.5	1.5	1
Control.....	2.5	2	1.5	2	1.5	1.5	1	1
Alanine								
Exptl.....	1.5	2.5	1	1.5	2	2.5	2	2.5
Control.....	2	2	1.5	2	2	2.5	2	2
Leucine								
Exptl.....				T	.5	1	.5	2
Control.....				0	1	1	.5	.5

<sup>1</sup> Each period represents 48 hours and the urine was made to 300 ml. The phenol system was used for developing the paper chromatograms.

nature of the tissue—'carcass,' 'kidney' or 'accessory sex gland' types. Moreover, the influence of dosage and duration of androgen (the concentration in the blood and tissues) must be borne in mind for the depletion of both carcass protein and fat was exacerbated by the intensity of treatment.

The loss in carcass fat on prolonged treatment may be due to an increase in energy metabolism due either to increased activity of the animal, therefore, a second-



ary phenomenon, or it may represent the cost of reshuffling the protein from the carcass to the internal organs. The nature of the demand, in any event, is such that it is not compensated for by an increase in appetite. Furthermore, the ability of the androgen to stimulate a utilization of the carcass fat occurs readily only in those animals that had or would have had a higher than normal deposit of carcass fat as a result of prolonged time after castration. In those animals that had a normal or subnormal amount of fat the utilization of the carcass fat did not occur until the nitrogen retention effect of the androgen had worn off. Thus, the nutritive state of the organism is a factor.

TABLE 14. TWO DIMENSIONAL AMINO ACID CHROMATOGRAMS OF HYDROLYZED AND UNHYDROLYZED URINE<sup>1</sup>

AMINO ACID	HYDROLYZED <sup>2</sup>		UNHYDROLYZED <sup>2</sup>	
	Exptl.	Control	Exptl.	Control
Aspartic acid.....	3	3	4	3
Glutamic acid.....	4	3	8	6
Glycine.....	5	6	10	10
Serine.....	2	1	3	3
Alanine.....	3	2	8	6
Alpha amino-butyric acid.....			1	0
Valine.....	2	1	5	3
Leucine.....	2	1	6	4
Phenylalanine.....			1	1
Threonine.....	1	T	2	2
Tyrosine.....			1	1
Arginine.....			1	1
Lysine.....	T	T	2	1
Proline.....			1	0
Histidine.....	1	1	1	2
Methionine sulfoxide.....	T	T	2	2
Citrulline.....	T	T	1	1
Taurine.....	3	5	8	10

<sup>1</sup> The chromatograms were first developed with phenol then with an equal mixture of collidine and 2,4 lutidine.

<sup>2</sup> The 48-hour urine sample was diluted to 300 ml., then one ml. was pipetted into a 10-ml. glass ampule, one ml. concentrated hydrochloric acid added, the tube sealed and placed in an electric oven at 120°C. for 24 hours. The contents were removed, concentrated to dryness, the residue dissolved in  $\frac{1}{2}$  ml. distilled water and 50  $\mu$ l. transferred to the paper chromatogram.

<sup>3</sup> A 20-ml. Aliquot of the 48-hour urine sample was concentrated to dryness on the steam bath and then made to one ml. of which 50  $\mu$ l. was transferred to the paper chromatogram.

The nearly identical amino acid composition of the tissues before and after androgen stimulation indicates that structurally at least the newly formed material is identical to that already present in the organ (cf. 14). This probably is not too surprising since all of the tissues in confirmation with previous chemical and microbiological (cf. 15) determinations have nearly identical amino acid compositions.

The inability to detect a marked decrease in amino acid excretion in the urine seems to indicate that the retention of the small threshold amounts of the amino acids is not necessary for the protein anabolic effect of the androgen. The synthesis of the new protein is accomplished by diverting the amino acids from oxidative processes as further indicated by a parallel decrease in urea excretion (1, 2).

The production of 'rusting' of the hair of the androgen-treated rats has been repeatedly observed in this laboratory. This phenomenon would suggest a pantothenic acid deficiency but the other aspects of such a vitamin deficiency except some bloody crusting of the eyes were not evident. Indeed the food intake of the rats fed *ad libitum* increased. It is well recognized, however, that pantothenic acid and several of the other B vitamins are necessary for protein metabolism and the formation of new tissue is accompanied by a retention of these vitamins to be incorporated with the newly formed tissue. It is possible, therefore, that the effect observed in the androgen-treated rats is an indication of mild or local pantothenic acid deficiency.

TABLE 15. TWO DIMENSIONAL AMINO ACID CHROMATOGRAMS OF TISSUES<sup>1,2</sup>

-AMINO ACID	LIVER				KIDNEY				SVPR.	MUSCLE	
	Protein <sup>3</sup>		Whole Tissue		Protein <sup>3</sup>		Whole Tissue		Protein <sup>3</sup>	Protein <sup>3</sup>	
	E	C	E	C	E	C	E	C	E	E	C
Aspartic acid.....	7.5	8	7.5	8	9	8.5	9	9	8	9	9
Glutamic acid.....	7	7	8.5	8	7	7.5	8	7.5	8	9	8
Glycine.....	6	6	7	7	6	6.5	7	7	7	7	6
Serine.....	5.5	6	5.5	5	5	5.5	6	5.5	7.5	6	6
Alanine.....	6.5	7	7	7	7	7	7	6.5	6	8	7
Valine.....	4.5	5	5	5	5	5	6	5	4	5	5
Leucine.....	7	7	8	8	6.5	7.5	8	8	6.5	8	8
Threonine.....	2	2	2.5	2	1	2	2	:	2	2	2
Tyrosine.....	1.5	2	2.5	2	1	1.5	2	2	2	1	1
Phenylalanine.....	1.5	2	1.5	2	1	1.5	2	2	2	1	2
Arginine.....	3	3	3	4	3	2.5	3	2.5	3.5	4	4
Lysine.....	3	3	3.5	4	3	2.5	3	2.5	4	3	4
Proline.....	+	+	+	+	+	+	+	+	+	+	+
Histidine.....	1	1	1	1	1	1	1	1	1	1	1
Taurine.....							2	2			
Hydroxylysine.....							1	1		1	1

<sup>1</sup> Amino acids were partitioned as for the urine; cf. table 14.

<sup>2</sup> Tissues were hydrolysed as follows: 50 mg. of the dried and powdered tissue was placed in a 10 ml. glass ampule, one ml. of 10 per cent hydrochloric acid was added, the ampule sealed and placed in an autoclave for 10 hours at 15 pounds pressure, then filtered through a sintered glass filter with suction, made to 5 ml. and 25  $\mu$ l. transferred to the paper chromatogram.

<sup>3</sup> Protein precipitated by trichloroacetic acid.

In any event the relationship of the protein anabolic effect of the androgens to vitamin requirements warrants detailed exploration and may provide an answer to the 'wearing off' effect.

#### SUMMARY

Castrated adult rats brought into body weight equilibrium and injected with testosterone propionate deposited protein in the carcass, seminal vesicles and prostates, liver and kidney in order. If, however, the intensity of treatment either by increased dosage or duration of treatment, was increased then the carcass began to lose not only fat but also protein which apparently was diverted to primarily the ac-

cessory sex organs and at a smaller extent the kidneys for these organs continued to grow. Furthermore, if the rats had a relatively high carcass fat content, the androgen caused a rapid reduction to the normal level. The above effect occurred in castrated and also normal rats fed *ad libitum*. Moreover, the androgen produced an increase in appetite of the castrated rats after 60 days of injection but not in the normal rats.

The qualitative and quantitative amino acid composition of the skeletal (gastrocnemius) muscle, liver, kidney and seminal vesicles and prostate were not changed by the androgen. Furthermore, the amino acid composition of each tissue was very similar. The total protein, non-protein nitrogen and amino acid nitrogen of the plasma of rats injected for 13 days with one mg/day of testosterone propionate were identical to those of control rats. The total amino acid and the partition of the urinary amino acids by paper chromatography showed no marked changes due to the androgen. A hypothesis for the mechanism of action of androgens on tumors is presented.

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# INFLUENCE OF FAT IN THE DIET UPON NITROGEN BALANCE AND LIVER REGENERATION<sup>1,2</sup>

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THE experiments of Goldschmidt, Vars and Ravdin (1, 2) on factors conditioning liver injury by certain hepatotoxic agents suggested that increased increments of hepatic lipid increased degeneration and necrosis. These authors recommended a diet high in carbohydrate and protein and low in fat prior to exposure to hepatotoxic agents to minimize liver injury. Such a diet leads to minimal lipid deposition in the liver and to maximal lipotropic action in livers containing an excess of lipid. No data were available from their experiments as to the effect of a diet containing varying amounts of fat on liver regeneration. The results reported in the present paper indicate that moderate amounts of fat in the diets of rats, which had been subjected to protein depletion and partial hepatectomy, does not impede regeneration of the liver parenchyma.

We have obtained data, in the rat, on the influence of varying amounts of dietary fat on the composition of the protein-starved and regenerating liver, and on the nitrogen balance. Also included are experiments in which the effect of the presence or absence of alpha-tocopherol acetate as part of the diet is assessed.

## METHODS

Young adult, male, Wistar rats, weighing approximately 250 grams, were placed into groups of six and maintained in individual cages. Body weight and food intake were recorded daily. Room temperature was maintained at approximately 27°C.

The composition of the various diets is given in table 1. Preoperatively, the animals were kept for a standard period of 2 weeks on a non-protein, 3 per cent fat (G-2) diet. During the 2-week period following 70 per cent partial hepatectomy, the control groups of animals were fed diets, *ad libitum*, containing 3 per cent fat and 0 or 10 per cent protein (casein). Other groups received 10 or 30 per cent fat diets so compounded and fed that these animals received calories, vitamins, minerals and nitrogen in total amounts equal to that of the control animals. In some of the re-

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<sup>3</sup> Harrison Fellow in Surgical Research 1948-1949.

<sup>4</sup> Harrison Fellow in Surgical Research 1947-1948.

alimentation experiments (*exper.* 33, 35) and in experimental group 33B, the 10 and 30 per cent fat-fed animals received total grams of food equal to that of a control group which were fed a 3 per cent fat, 10 per cent casein diet (G-6) *ad libitum*. Thus the nitrogen and caloric intake of these animals was greater than that of the control group. Rats in group 13A received an 18 per cent casein, 3 per cent fat diet *ad libitum*.

All diets contained either cod-liver oil or one drop daily of oleum percomorphum which contained 1250 units of vitamin A and 180 units of vitamin D. In the vitamin E experiments, the preoperative diets were tocopherol-free. The postoperative supplement was 5 mg. daily of alpha-tocopherol acetate, orally, in one drop of oleum percomorphum. All diets were prepared fresh prior to actual use, and the excess was refrigerated.

TABLE 1. COMPOSITION OF VARIOUS DIETS

	G-2	G-6	F-4	RF-1 <sup>1</sup>	RF-2 <sup>2</sup>	RF-3 <sup>1</sup>	RF-4 <sup>3</sup>	F-1	F-2	F-3
Sucrose.....	91	81	48.9	81	81	48.9	48.9	84	62.1	73.1
Casein <sup>4</sup> .....	0	10	13.2	10	10	13.2	13.2			10.9
C.L.O.....	3	3	3					3	3	3
Jones salts no. 12 <sup>4</sup> .....	4	4	5.3	4	4	5.3	5.3	4	5.3	4
Cellu flour.....	2	2	2.6	2	2	2.6	2.6	2	2.6	2
Mazola.....			2					2	2	2
Crisco.....			25					5	25	5
Low toc. fat <sup>5</sup> .....				3	3	30	30			
Cal. gm.....	3.91	3.91	5.18	3.91	3.91	5.18	5.18		5.18	

Control animals received diets containing a vitamin supplement composed of the following quantities per 10 gm. unit of food: thiamine, riboflavin and pyridoxine, 0.1 mg. each; nicotinic acid, 1.0 mg.; inositol, 6.0 mg.; p.a.b.a., 2.0 mg.; Ca pantothenate, 0.6 mg., and choline chloride, 20 mg.

<sup>1</sup> Added 5 mg.  $\alpha$  tocopherol acetate in one drop oleum percomorphum daily. <sup>2</sup> Added one drop oleum percomorphum daily. <sup>3</sup> Fat free casein #453, Casein Manufacturing Co., Bainbridge, N. Y. <sup>4</sup> Jones, J. H. and C. Foster. *J. Nutrition* 24: 245, 1942. <sup>5</sup> Vit. E Free Lard: Distillation Products, Inc., Rochester, N. Y.

The experimental procedures at operation and sacrifice, the methods of excreta collection, histological preparations, and analysis for nitrogen, lipids, glycogen, liver solids and water, serum proteins, and the methods of calculation were those previously reported (3-5). The lipid values given in table 2 are from the petroleum-ether soluble portion of the alcohol-ether extract from the pooled livers.

The N balance studies were begun 4 days preoperatively and continued throughout the postoperative period, during which time the animals were housed in individual metabolism cages. Rats in the re-alimentation experiments were not subjected to any operative procedure, but were otherwise subjected to the same procedures and analyses as the animals in the other groups.

## RESULTS

The data are summarized in table 2. The data in experiments 13A, 14A and 19 have been previously reported (3-5).

From the data in table 2 and the histograms in figures 1 and 2, it may be seen that

TABLE 2. SUMMARY OF EXPERIMENTAL DATA. EFFECT OF VARYING AMOUNTS OF DIETARY FAT ON N BALANCE AND LIVER COMPOSITION

EXPER.	NO. OF RATS	POSTOP. DIET			POSTOP. N <sup>1</sup> BAL-ANCE	BODY WEIGHT		LIVER COMPOSITION					SACRIFICE SERUM PROTEIN
			Fat	N <sup>1</sup> intake		Initial	Gain or Loss	Postop. increment		Sacrifice values			
								Protein <sup>1</sup>	Mass <sup>1</sup>	Lipids	Gly-cogen	H <sub>2</sub> O	
			%			gm.	gm.			%	%	%	gm. %
19	4	G-2	3	0	-.28 ±.02	250 ±5	-27	.17 ±.01	1.34		5.4	70.0	4.2
31A	4	F-1	10	0	-.25 ±.02	243 ±10	-19	.16 ±.01	1.38	8.2	7.9	70.0	4.8
32A	4	F-2	30	0	-.20 ±.05	255 ±4	-21	.15 ±.03	1.24	9.3	7.3	69.8	4.4
14A	6	G-6	3	.77 ±.10	+ .24 ±.06	265 ±14	+17	.30 ±.03	2.00		8.2	69.5	5.8
66A	4	RF-3	30	.74 ±.03	.15 ±.04	265 ±11	24	.31 ±.02	1.16	5.6	0.7	70.0	5.4
66B	4	RF-4	30	.74 ±.02	.18 ±.06	267 ±7	26	.31 ±.02	1.19	6.4	0.4	70.9	5.4
65A	4	RF-1	3	.83 ±.06	.31 ±.06	239 ±4	32	.33 ±.03	2.04	7.6	4.5	69.2	5.1
65B	4	RF-2	3	.84 ±.05	.34 ±.08	257 ±18	33	.33 ±.02	1.84	6.7	4.9	71.0	5.2
47	5	F-3	10	.82 ±.02	.31 ±.03	248 ±5	30	.35 ±.03	1.21	7.9	0.4	70.6	5.2
33C,D	9	F-4	30	.82 ±.02	.21 ±.02	250 ±7	38	.36 ±.02	1.52	5.8	2.7	70.4	5.3
46	5	No- choline F-4	30	.82 ±.02	.27 ±.04	250 ±6	23	.38 ±.03	2.31	31.4	0.4	53.3	5.4
68A	5	G-6	3	.98 ±.09	.27 ±.05	245 ±10	34	.34 ±.03	2.01	4.1	5.8	70.3	5.2
68B	6	F-4	30	.96 ±.03	.34 ±.04	245 ±10	40	.36 ±.04	1.60	4.5	<0.1	71.1	5.8
69A	5	RF-3	30	.96 ±.04	.28 ±.02	247 ±11	40	.35 ±.02	1.53	5.3	<0.1	71.6	5.5
69B	5	RF-4	30	.96 ±.05	.29 ±.03	248 ±12	38	.36 ±.03	1.62	5.6	<0.1	71.1	5.2
67	6	RF-3	30	.96 ±.11	.24 ±.09	294 ±9	38	.36 ±.05	1.70	5.6	3.6	70.6	5.3
33B	5	F-4	30	1.03 ±.05	.33 ±.04	266 ±7	59	.42 ±.03	2.07	4.9	4.0	70.2	5.9
13A	4	G-1	3	1.47 ±.17	.45 ±.08	245 ±18	30	.37 ±.04	2.07	4.8	5.9	69.2	5.9

Realimentation Experiments<sup>2</sup>

7A	5	G-2	3	0	—	259 ±12	-18	.37 ±.03	2.69	8.6	6.0	70.3	
31	5	F-1	10	0	-.26 ±.01	253 ±7	-14	.34 ±.002	2.60	7.3	7.2	70.9	4.8
32	6	F-2	30	0	-.25 ±.01	251 ±9	-12	.36 ±.03	2.76	11.2	7.0	69.0	4.1
34	4	G-6	3	.82 ±.07	+ .29 ±.04	258 ±6	+27	.50 ±.02	3.27	5.0	6.7	70.4	5.6
35	5	F-3	10	.94 ±.07	.32 ±.04	259 ±5	44	.55 ±.03	3.27	5.1	4.8	70.3	5.9
33	4	F-4	30	1.09 ±.06	.43 ±.01	271 ±5	61	.59 ±.05	3.08	6.0	7.2	70.2	5.6

<sup>1</sup> Values expressed in gm. per 100 gm. of initial body weight.<sup>2</sup> Sacrifice values.

groups of rats fed 30 per cent fat diets and limited in total calories, nitrogen, vitamins, and minerals equal to that of *ad libitum*-fed animals receiving 3 per cent fat diets, showed the following results as compared to the 3 per cent fat-fed controls: 1) Greater body weight gains when the diet contained protein, and less body weight losses when the diet contained no protein. 2) Slightly less liver protein increments when the diet contained no protein, but slightly greater liver protein increments in each series in which the diet contained protein. The difference between the means of group 33CD and 68AB is about  $2\frac{1}{2}$  times the standard error of the difference between the means, i.e. its probability of occurring merely by chance is about one in 80. Difference between other groups is not 'significant.' 3) Slightly greater N conservation at the higher levels of protein intake, but significantly less positive N balances at the lower levels

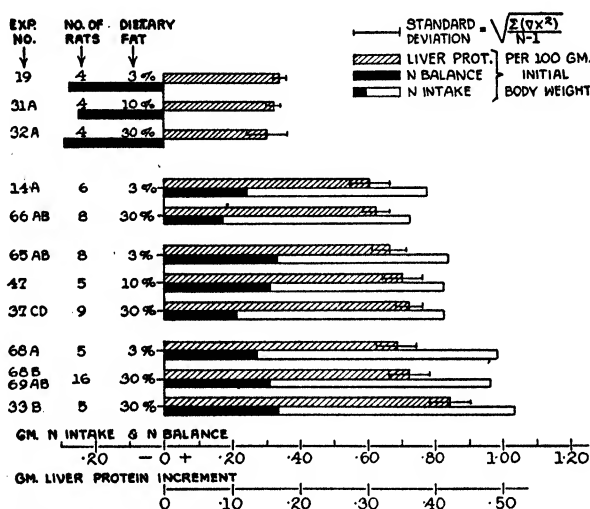


Fig. 1. COMPARISON OF THE NITROGEN INTAKE, balance and liver protein increment in rats fed isonitrogenous, isocaloric diets containing 3, 10 and 30 per cent fat.

of protein intake. 4) Similar percentages of liver fat and water, and serum proteins at sacrifice. 5) Group 46, which received no choline in the diet, had less N conservation and a smaller body weight gain. The livers of these animals were heavily infiltrated with fat, yet contained a slightly greater amount of new liver protein than other similar, but choline-fed animals. 6) Smaller amounts of liver glycogen. This can be explained by the time of food consumption in relation to sacrifice. The animals receiving the higher fat diets found these more palatable and consumed their weighed amount of food shortly after it was offered. The 3 per cent fat-fed animals, on the other hand, consumed food more slowly and had access to food up to the hour of sacrifice and 7) results were similar whether or not the diet was deficient in vitamin E. This was true of both the 3 per cent and the 30 per cent fat-fed animals. Similarly, no difference was noted in the results whether the fat offered was cod-liver oil, a combination of cod-liver oil, Mazola oil and Crisco, or lard.

When the same diets were fed, with the 30 per cent fat-fed animals (*group 33B*) limited only in total grams of food intake equal to that eaten by the 3 per cent fat-fed animals (*group 14A*), the animals receiving the higher amounts of fat had significantly better N balances and gains of liver protein and body weight. For N balances, the difference between the means is greater than  $3\frac{1}{2}$  times the standard error of the difference between the means. For liver protein increments, the difference is more than  $6\frac{1}{2}$  times the standard errors. In both the 3 per cent and the 30 per cent fat-fed animals, the body weight gains, N conservation and new liver protein formation increased as the dietary protein intake was increased.

Histological sections showed no noteworthy abnormality except in the livers of the choline deficient animals, where there was marked fatty infiltration. Clinically, the animals receiving the greater amounts of fat looked healthier, were more active, and had better appetites.

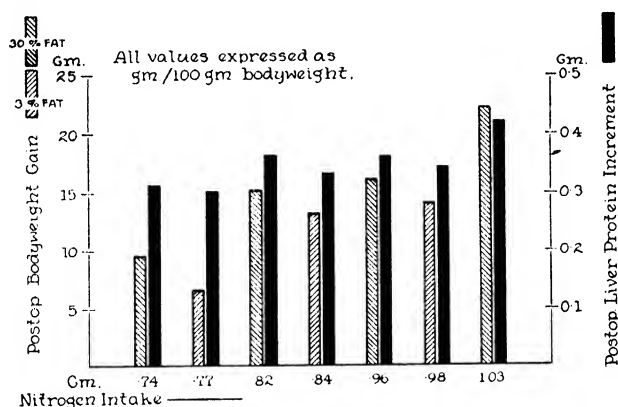


Fig. 2. COMPARISON OF THE POSTOPERATIVE WEIGHT gain and liver protein increment at varying nitrogen intakes with 3 and 30 per cent fat diets.

#### DISCUSSION

In 1939, Goldschmidt, Vars and Ravdin (1) demonstrated that in rats the degree of liver damage following chloroform anesthesia decreased with increasing amounts of dietary protein intake prior to anesthesia, and increased with increasing amounts of lipids present in the liver at the time of anesthetization. These results were confirmed in experiments on dogs by Miller and Whipple (6) who found that liver injury following chloroform anesthesia increased in extent as the protein stores were depleted.

The beneficial effects of high caloric and high protein diets were further supported by the recent investigations of Vars, Gurd and Ravdin (3-5) who found the increment of liver protein following partial hepatectomy in rats was increased with increasing amounts of protein in the postoperative diet. Campbell and Kosterlitz (7) demonstrated that the quantity of non-glycogen, non-lipid, liver solids (NNS) varied directly with the log of the protein intake, and that the nutritive value of protein was greatly decreased when the caloric intake was restricted. The data presented in this paper



likewise demonstrate the beneficial effects of a high protein and high caloric intake. The latter differs in the fact that the high caloric intake was achieved by the use of fat. The results indicate that moderate amounts of fat in the diet are not deleterious in liver regeneration in the rat following partial hepatectomy. The data demonstrate that the fat concentration of the liver is no greater after the ingestion of diets containing 30 per cent fat than after the ingestion of diets containing 3 per cent fat, provided there are adequate amounts of protein and other lipotropic substances in the diet.

Forbes, Swift and associates (8-10) working with growing and adult rats have reported that liberal amounts of fat in the diet resulted in superior energy utilization of the diet as a whole. Increased body weight, increased body gains of nitrogen, fat and energy, and decreased heat production resulted as the fat content of isocaloric diets was increased from 2 per cent to 30 per cent with all nutrients furnished in optimum quantities.

Deuel and his associates (11, 12) have reported that rats receiving diets containing up to 50 per cent fat grow more rapidly, reach a greater final weight, attain sexual maturity earlier, are more fertile, raise larger young, and have a greater capacity for exhausting work than control animals fed diets containing no fat. Optimal results were obtained with diets containing 20 to 30 per cent fat.

Schwimmer and McGavack (13) reported metabolic studies on Army personnel fed isocaloric diets at 900 calorie level with each diet supplying 6 gm. of protein nitrogen. The group receiving a diet containing 30 per cent fat had greater N conservation and utilization than did the groups receiving 20 per cent or 10 per cent fat in the diet. Hoagland and associates (14, 15), using the criteria of weekly liver function tests, weight gain or loss and days to recovery as measured by clinical evaluation, found no harmful effects of moderate amounts of fat in the diet fed patients with infectious hepatitis.

Clayton and Bauman (16) reported that mice and rats were more resistant to 2,4-dinitrotoluene as evidenced by better growth and better survival, when fed diets containing 5 or 30 per cent fat than when fed isocaloric diets containing 0.46 per cent fat, while Hove *et al.* (17) reported that high fat diets decreased resistance to anoxic anoxia in rats subjected to decreased oxygen pressure.

Hove (18) reported that a daily supplement of one mg. of *d*, alpha-tocopherol prevented the marked weight loss, muscle dystrophy, testicular atrophy, stomach ulcers, and tooth pigmentation which occurred in pair-fed control rats beginning after about ten weeks on a 5 per cent casein diet. Hove and Harris (19) reported that a daily supplement of one mg. of *d*, alpha-tocopherol increased the utilization of casein protein for the growth of young rats when casein levels in the diet were between 6 and 12 per cent.

In the vitamin E experiments reported herein, the results were essentially the same whether or not the postoperative diet was deficient in vitamin E. These results were obtained with diets containing no protein with 3 per cent fat and 13.2 per cent casein with 30 per cent fat.

#### CONCLUSIONS

Following 70 per cent partial hepatectomy in previously protein-depleted rats, the increments of liver protein and body weight and N conservation increased and

the liver lipid concentration decreased as the postoperative dietary protein was increased.

The liver composition was essentially identical; the N conservation, slightly less; and the body weight gains significantly greater in 10 per cent and 30 per cent fat-fed animals as compared to 3 per cent fat-fed animals when the diet contained equal total amounts of calories, N, vitamins and minerals. When 30 per cent fat-fed animals were limited only in total grams of food intake, equal to that of *ad libitum*, 3 per cent fat-fed animals, the 30 per cent fat-fed animals had significantly greater N balance and increments of liver protein and body weight. A group of animals fed a choline deficient diet had a liver protein increment equal to that of pair-fed controls, even though their livers were heavily infiltrated with fat. Moderate amounts of fat in the diet increased the palatability of the diet and resulted in a greater intake of all food components when fed *ad libitum*.

In the 2-week postoperative period, there was no essential difference in the body weight gain, N balance or liver composition of rats on a vitamin E deficient, 10 or 13.2 per cent casein, 3 or 30 per cent fat diet and pair-fed animals receiving a daily oral supplement of 5 mg. of *d*-tocopherol acetate. Under the conditions of these experiments, moderate amounts of fat in the diet were not deleterious in experimental liver regeneration. The advisability of severely limiting dietary fat in the diets of patients with liver injury is not supported by the results of our studies.

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# ROLE OF ADRENAL CORTEX IN LIVER REGENERATION<sup>1,2</sup>

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THE following experiments were undertaken to investigate the part that the adrenal cortex plays in influencing protein metabolism and liver regeneration. It is known that the steroids of the adrenal cortex promote the conversion of glucose to liver glycogen (1) and the catabolism of liver protein to glucose (2). These processes are depressed in animals with adrenal insufficiency. In normal animals, fatty livers can be produced by various methods, but, in adrenalectomized animals, the deposition of fat in the liver is inhibited (3-5).

Previous work in this laboratory has shown that following partial hepatectomy the protein-depleted rat will regenerate a constant amount of new liver depending on the type and amount of diet fed postoperatively (6). The protein content of the liver, rather than the organ's total mass, was used as the measure of effective liver size (7).

## EXPERIMENTAL PROCEDURES

Young adult male Wistar rats weighing approximately 250 grams were used throughout this investigation. For two weeks prior to surgery they were fed *ad libitum* a synthetic nonprotein diet to insure as little variation as possible in their nutritive condition. One group of 10 rats were not depleted, and for one week were fed a similar diet to which was added 18 per cent casein. Compositions of the diets are given in table 1.

The operation of partial hepatectomy,  $69.4 \pm 1.34$  per cent was essentially that described by Higgins and Anderson (8) with modifications as reported by Gurd, Vars, and Ravdin (6). When bilateral adrenalectomy was also performed at the same time, the glands were removed through the abdominal approach.

Prior to operation all of the animals were given subcutaneously 5.0 ml. of 5 per cent glucose in isotonic saline. Immediately after the operation and again in 6 hours the protein-depleted rats, except in *experiment 43*, were given 0.5 ml. of adrenal cortical extract. Following the operation the animals were placed on the various treatments as indicated in tables 2 and 3. All animals were treated for 2 weeks except for three groups which were observed for only 2 days postoperatively.

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For 4 days prior to operation and throughout the postoperative period the animals were housed in individual metabolism cages. The adrenalectomized animals were given a one per cent saline solution to drink, and in many experiments the total intake was recorded. The synthetic diets were freshly prepared prior to each experiment and were kept under refrigeration until used. Body weight and the amount of food eaten was recorded daily. Urine and feces were collected daily, and analyzed for nitrogen. Details of collection and of analytical methods were reported previously (9).

On the 14th postoperative day the rats were sacrificed in the same order and time at which they were operated. Under sodium amytal anesthesia the abdomen was opened, and blood was withdrawn from the inferior vena cava for serum protein and chloride analysis.

TABLE 1. COMPOSITION OF EXPERIMENTAL DIETS

	DIET				
	G-2	G-1	G-6	F2	F4-F5
Sucrose.....	91	73	81	62.1	48.9
Casein <sup>1</sup> .....	0	18	10	0	13.2
Cod liver oil.....	3	3	3	3	3
Salts 185.....	4	4	4	5.3	5.3
Celluloflour.....	2	2	2	2.6	2.6
Mazole.....	0	0	0	2	2
Crisco.....	0	0	0	25	25

Each animal received a vitamin supplement composed of the following: thiamine, riboflavin and pyridoxine, 0.1 mg. each; nicotinic acid 1.0 mg.; inositol 6 mg.; Ca-pantothenate 0.6 mg.; p.a.b.a. 2.0 mg.; choline chloride 20 mg. In diets G-1, G-2, G-6, the above was supplied per 10 gm. unit of food. In diets F-2, F-4, the above was supplied per 7.5 gm. unit of food, with double the amount of choline chloride. Diet F-5 was identical with F-4 except that no choline chloride was supplied in the vitamin mixture.

<sup>1</sup> Fat-free casein no. 453, Casein Manufacturing Co., Bainbridge, New York.

The large blood vessels between liver and diaphragm were divided and the liver removed. At both operation and sacrifice the removed liver was freed of excess blood. Samples were taken for solids determinations and histology. The remainder of the liver was pooled for nitrogen, glycogen, and lipid determinations. The methods and outline of analyses and methods of computation were presented in a previous publication (6).

Sections of kidney, testis, and thymus were taken for microscopic examination. Perirenal fat was sectioned serially for aberrant adrenal tissue.

#### RESULTS

The effect of removing both adrenal glands on the amount of new liver protein regenerated is presented in table 2, together with data from rats hepatectomized only. In both the protein-depleted rats (*experiments 40, 42, 43*) and the group (*experiment 37*) fed 18 per cent casein preoperatively, the amount of liver protein regenerated was markedly decreased in the adrenalectomized rats. The depleted ani-

TABLE 2. COMPOSITION OF REGENERATING LIVER AFTER PARTIAL HEPATECTOMY AND SIMULTANEOUS ADRENALECTOMY IN NORMAL AND PROTEIN-DEPLETED RATS<sup>1,2</sup>

EXPER. NO.	OPER.	NO. OF RATS	DIET		BODY WEIGHT		SERUM PROTEIN		LIVER COMPOSITION										NITROGEN <sup>2</sup>	
			Pre-Oper.	Post-Oper.	Init.	Oper.	Sacr.	gm. %	Mass <sup>3</sup>		Solids		Glycogen		Lipids		Proteins <sup>4</sup>		Intake	Bal.
					gm.	gm.	gm.		Oper.	Sacr.	Oper.	Sacr.	Oper.	Sacr.	Oper.	Sacr.	Oper.	Sacr.	gm.	gm.
37	HA	10	G1	G2	358 ±11	258	203	4.6 ±0.3	3.82 ±0.40	1.60 ±0.34	30.3 ±2.0	27.2 ±1.3	4.8 ±0.3	2.5 ±0.1	4.7 ±0.2	5.7 ±0.02	0.26 ±0.08	0.046 ±0.008	0	-0.36 ±0.03
23C	H	6	G1	G2	254 ±7	254	198	4.9 ±0.3	4.15 ±2.1	2.57 ±0.17	31.1 ±0.4	30.4 ±0.9	7.3 ±0.3	9.0 ±0.1			0.33 ±0.05	0.111 ±0.017	0	-0.44 ±0.04
54	HA	5	G1	G2	250 ±8	250	231	4.7 ±0.2	4.02 ±0.86	1.47 ±0.15	30.9 ±0.6	27.1 ±0.9	5.6 ±0.1	0.1 ±0.01	4.3 ±0.1	8.6 ±0.05	0.27 ±0.03	0.051 ±0.010	0	-0.11 ±0.02
43	HA	2	G2	G2	249	198	198	3.8	2.36	1.10	29.9	28.7	4.9		7.0	0.39	0.16	0.04	0	-0.05
42	HA	6	G2	G2	246 ±5	192	192	3.8 ±0.1	2.79 ±0.33	1.22 ±0.11	30.7 ±1.2	30.4 ±2.2	6.0 ±0.2	1.5 ±0.01	6.4 ±0.1	11.0 ±0.05	0.36 ±0.03	0.19 ±0.02	0	-0.04 ±0.004
2	H	5	G2	G2	242 ±15	194	186	4.4	2.95 ±0.34	1.58 ±0.18	29.1 ±1.1	28.8 ±0.3	11.2 ±0.3	3.6 ±0.1	5.7 ±0.1	9.6 ±0.03	0.41 ±0.01	0.02 ±0.005	0	
40	HA	5	G2	G2	251 ±3	212	178	4.4 ±0.1	3.00 ±0.23	1.57 ±0.39	31.5 ±0.4	28.0 ±2.3	6.4 ±0.1	7.9 ±0.1	5.6 ±0.1	6.4 ±0.03	0.40 ±0.04	0.23 ±0.05	0	-0.25 ±0.04
19	H	4	G2	G2	250 ±5	198	170	4.2	2.88 ±0.39	2.34 ±0.30	30.6 ±2.1	30.1 ±2.5	5.9 ±0.1	3.6 ±0.1			0.42 ±0.03	0.17 ±0.02	0	-0.28 ±0.02
36	HA	4	G2	G6	255 ±4	208	194	4.6 ±0.2	2.85 ±0.26	1.82 ±0.15	30.1 ±0.6	26.7 ±1.2	7.8 ±0.1	3.1 ±0.1	6.8 ±0.1	4.2 ±0.06	0.39 ±0.02	0.29 ±0.02	0.55 ±0.05	+0.16 ±0.08

14	H	6	G2	G6	14	265 ±14	224	241	5.8 ±0.2	2.60 ±0.23	2.78 ±0.34	29.4 ±1.0	30.6 ±0.8	6.9	8.2	5.8	5.4 ±0.38	0.42 ±0.03	0.30 ±0.03	0.77 ±0.09	+0.24 ±0.06
41	HA	3	G2	F2	14	250 ±2	214	191	4.4 ±0.2	2.90 ±0.19	1.90 ±0.29	30.5 ±0.3	29.3 ±1.4	7.8	4.3	9.6	9.2 ±0.44	0.27 ±0.07	0.13 ±0.03	0 ±0.02	
32A	H	4	G2	F2	14	255 ±5	204	184	4.4 ±0.3	2.57 ±0.55	2.04 ±0.17	28.2 ±1.2	30.2 ±1.4	5.2	7.3	5.5	8.4 ±0.36	0.26 ±0.05	0.15 ±0.03	0 ±0.05	
39	HA	5	G2	F4	14	249 ±2	206	217	4.9 ±0.3	2.71 ±0.42	1.99 ±0.32	30.9 ±0.9	28.4 ±1.3	8.7	3.9	7.0	5.1 ±0.42	0.35 ±0.06	0.22 ±0.03	0.74 ±0.09	
33C.D.	H	9	G2	F4	14	250 ±8	210	248	5.3 ±0.2	2.60 ±0.18	2.30 ±0.14	30.0 ±0.5	29.4 ±0.9	5.2	1.1	5.8	4.9 ±0.35	0.47 ±0.04	0.36 ±0.02	0.82 ±0.02	
73	HA	5	G2	F5	14	253 ±7	200	240	4.6 ±0.2	2.85 ±0.11	3.22 ±0.27	31.5 ±1.6	35.7 ±2.3	9.2	3.2	5.6	14.6 ±0.35	0.45 ±0.02	0.34 ±0.02	0.90 ±0.09	
46	H	5	G2	F5	14	250 ±8	210	233	5.4 ±0.3	3.41 ±0.32	3.34 ±0.25	31.4 ±1.7	46.7 ±2.1	7.2	0.4	8.1	31.4 ±0.40	0.50 ±0.02	0.38 ±0.03	0.82 ±0.02	
38	HA	7	G2	G1	14	251 ±7	202	239	5.0 ±0.4	2.82 ±0.44	2.27 ±0.21	30.4 ±1.1	27.3 ±0.7			4.7	5.0 ±0.38	0.45 ±0.04	0.33 ±0.03	1.42 ±0.03	
13	H	4	G2	G1	14	254 ±18	199	239	5.8 ±0.3	2.36 ±0.19	2.78 ±0.27	29.4 ±0.5	30.8 ±0.7	6.3	5.8		0.39 ±0.03	0.49 ±0.04	0.37 ±0.04	1.47 ±0.17	

<sup>1</sup> Values shown are averages for each experimental group. Where individual values were determined, the standard deviation is shown. S.D. =  $\pm \sqrt{\sum(\Delta X)^2 / n-1}$ .

<sup>2</sup> Values expressed in gm/100 gm. initial body weight. <sup>3</sup> All doubly operated rats received s.c. injection of 5 ml. 5 per cent glucose-saline immediately before operation and were offered 1 per cent NaCl solution to drink throughout the experiment. All adrenalectomized groups except experiments 37, 54 and 43 received s.c. injection of 0.5 ml. whole adrenal extract following operation and 6 hours later.

TABLE 3. EFFECT OF ADRENAL STEROIDS UPON COMPOSITION OF REGENERATING LIVER AND NITROGEN BALANCE AFTER PARTIAL HEPATECTOMY AND ADRENALECTOMY IN RATS<sup>1</sup>

EXPER.	OPER.	NO. OF RATS	THERAPY	H <sub>2</sub> O IN- TAKE ml.	BODY WEIGHT			SERUM		LIVER COMPOSITION										NITROGEN <sup>2</sup>						
					Initial	Oper.	Sacr.	Prot.	NaCl	Mass <sup>3</sup>		Solids		Glycogen		Lipids		Protein <sup>3</sup>		Intake	Bal.					
										gm.	gm.	gm. %	mg. %	Oper.	Sacr.	%	%	Oper.	Sacr.			%	%	Oper.	Sacr.	Regen.

Pre-op. Diet G-2 for 14 Days; Post-op. Diet for G-2 14 Days																						
50	HA	6	17 OH, 11 dehydro., 1 mg/day	370	252	210	188	3.7		2.97	1.88	30.3	27.8	0.4	6.5	6.5	5	0.40	0.28	0.16	0	-0.31
					±6		±0.2		±0.30	±0.24	±1.4	±0.4								±0.01	±0.02	±0.02
60	HA	5	11 dehydro., 1 mg/day	480	247	202	181	3.8	590	2.85	1.97	30.7	27.5	4.7	16.9	5.8	0.37	0.28	0.17	0	-0.32	
					±4		±0.3	±15	±0.33	±0.23	1.0	±0.9								±0.03	±0.02	±0.02
51	H	6	whole adrenal extract, 2 ml/day		249	202	171	4.0		2.86	2.20	31.0	30.1	18.1	17.1	16.3	0.39	0.28	0.16	0	-0.34	
					±2		±0.2		±0.32	±0.13	±1.0	±0.5								±0.02	±0.02	±0.01
45	HA	11	whole adrenal extract, 0.5 ml. X 7, 0.25 ml. X 7 day		250	201	175	3.8		2.65	1.56	30.1	26.0	4.8	17.1	16.3	5.4	0.36	0.28	0.17	0	-0.27
					±4		±0.3		±0.24	±0.20	±1.1	±1.0								±0.03	±0.03	±0.02
44, 48	HA	9	DCA, 5 mg. X 3, 2.5 mg. X 11 day		249	206	189	3.7		2.84	1.99	31.4	26.8	6.1	13.2	8.6	8	0.38	0.31	0.19	0	-0.27
					±5		±0.3		±0.49	±0.21	±1.2	±1.3								±0.05	±0.04	±0.05
52	HA	5	DCA 1/10 above	260	249	207	191	3.4		2.87	1.89	29.7	25.8	8.1	13.4	4.8	5.5	0.39	0.26	0.14	0	-0.24
					±3		±0.1		±0.25	±0.40	±0.7	±1.5								±0.01	±0.02	±0.02

Pre-op. Diet G-2 for 14 Days; Post-op. Diet G-6 for 14 Days

59	HA	6	11 dehydro, 1 mg/day	540	247 ±4	197	208	5.0 ±0.2	590	2.93 ±0.23	2.41 ±0.16	31.9 ±1.0	28.6 ±0.9	1.1 ±0.1	7.4 ±0.4	0.37 ±0.02	0.48 ±0.02	0.37 ±0.02	0.85 ±0.05	±0.01
56	HA	5	whole adrenal extract, 0.5 ml. X 7, 0.25 ml. X 7 day	280	250 ±6	202	226	4.7 ±0.3	620	2.68 ±0.26	2.50 ±0.33	29.9 ±0.3	28.4 ±0.8	7.5 ±0.5	8.4 ±0.4	0.38 ±0.01	0.41 ±0.02	9.29 ±0.03	0.81 ±0.06	±0.05
63	H	3	DCA, 5 mg. X 3, 2.5 mg. X 11 day	330	255 ±10	223	249	5.0 ±0.3	530	3.04 ±0.46	3.05 ±0.51	30.5 ±0.8	29.3 ±1.3	6.7 ±0.5	8.5 ±0.4	0.42 ±0.02	0.46 ±0.02	0.32 ±0.02	0.62 ±0.02	±0.01
55	HA	6	DCA, as above	690	250 ±5	203	240	4.8 ±0.1	535	2.85 ±0.21	3.35 ±0.34	30.4 ±0.5	28.6 ±0.6	9.5 ±0.5	5.4 ±0.2	0.37 ±0.03	0.49 ±0.03	0.38 ±0.03	0.85 ±0.06	±0.05
57	H	5	DCA, as above	960	244 ±2	200	211	4.6 ±0.3	525	2.90 ±0.19	2.86 ±0.10	30.7 ±1.0	28.1 ±0.2	17.7 ±0.2	6.4 ±0.3	0.39 ±0.02	0.51 ±0.03	0.39 ±0.03	0.71 ±0.10	±0.01
58	HA	4	DCA, as above	190	247 ±4	208	232	4.7 ±0.2	560	3.17 ±0.24	2.91 ±0.23	31.0 ±0.8	27.8 ±1.0	9.2 ±0.3	6.2 ±0.5	0.38 ±0.01	0.51 ±0.06	0.40 ±0.06	0.75 ±0.15	±0.01
61	HA	3	17 OH, 11 dehydro, P.D., 5 day Merck 9 day	330	247	210	216	5.4	580	2.58	2.24	30.3	29.1	4.6	8.1	0.40	0.40	0.28	0.69	±0.14
62	HA	2	as above, 14 day P.D.	440	270	238	255	5.5	570	3.29	2.53	31.6	28.2	6.7	1.4	0.42	0.44	0.32	0.71	±0.11
64	HA	3	as above, 14 day Merck	920	263	222	233	5.2	580	3.12	2.64	30.4	30.9	7.9	5.9	0.40	0.42	0.30	0.72	0.0

<sup>1</sup> See footnotes to table 2. <sup>2</sup> All groups except *experiments 57, 63, and 58* received 1 per cent NaCl solution to drink throughout the post-operative period.

<sup>3</sup> Values expressed in gm/100 gm. initial body weight.



imals regenerated only 60 per cent of the amount of liver protein as did the control rats. The group fed an 18 per cent casein diet had 40 per cent of the new liver protein values of rats with intact adrenals. Control animals (*experiment 2*) sacrificed 2 days following partial hepatectomy regenerated 0.095 gm. of new liver protein per 100 gm. of initial body weight. In contrast the untreated adrenalectomized rats (*experiments 43, 54*) formed only 0.05 and 0.04 gm. of new liver protein. Protein-depleted rats in contrast to well-fed animals exhibit a high mortality when subjected to the double operation and are given only saline-glucose subcutaneously and saline to drink. Therefore, all subsequent doubly operated depleted rats received cortical extract upon the day of operation. By treating a group (*experiment 42*) of adrenalectomized animals with 4 doses of 0.5 ml. of whole extract over the 2-day postoperative period, 0.082 gm. of new liver protein per 100 gm. of initial body weight was formed.

The only other significant finding was that in most groups the livers of the adrenalectomized animals had a higher water content and lower glycogen value. The plasma protein and chloride levels were not altered appreciably.

The effect of adrenalectomy on liver protein regeneration in animals fed diets of different protein content was investigated (*experiments 36, 38*). Here again, removal of the adrenals impaired the liver's regenerative power. Even with a much more positive nitrogen balance, such as observed in *experiment 38* as compared with the control group (*experiment 13*), there was less new liver protein formed.

The feeding of 30 per cent fat diets, F-2 and F-4 (*experiments 39, 41*) together with adequate choline, did not change the composition of the regenerating livers of adrenalectomized rats. When choline was removed from the 30 per cent fat ration (*experiment 73*) the regenerating liver contained less than half the total lipid found in rats with intact adrenals. The presence of extra fat in the diet did not retard liver protein regeneration.

After observing the retarding influence that adrenalectomy had on the regenerating liver, we investigated the effect of treating the partially hepatectomized rats with various adrenal cortical hormones (table 3). *Experiments 44, 45, 48, 50, 52, and 60* summarize the results obtained when the treated animals were fed the non-protein diet postoperatively, while in *experiments 55 to 64* inclusive a 10 per cent casein diet was fed. The nitrogen intake was kept fairly constant between the treated and control rats. The highest values of new liver protein formed were obtained in those rats (*experiments 44, 48, 55 and 58*) given daily subcutaneous injections of desoxycorticosterone acetate (DCA) in oil (Ciba). The dosage used, 5.0 mg. for 3 days and 2.5 mg. for the next 11 days, is much greater than is necessary for life maintenance. We therefore gave the animals in *experiment 52* one-tenth of that amount of DCA. They did better than the untreated animals, but not as well as the controls (*experiment 19*) or the groups treated with the larger dose. Another group of animals (*experiment 57*) with the adrenals intact were partially hepatectomized and given the larger dose of DCA daily. These rats regenerated an even greater amount of new liver protein.

It was noticed that these groups of DCA-treated animals (*experiments 55 and 57*) ingested excessive volumes of saline, averaging 690 ml. and 960 ml. per rat for the 14-day postoperative period as compared with the control values of approxi-

mately 200 ml. To determine the effect of the increased salt intake, another group of adrenalectomized rats (*experiment 58*) was treated with the same dose of DCA but were given plain water rather than saline to drink. These rats regenerated as much new liver protein as the group (*experiment 55*) given one per cent sodium chloride to drink.

By injecting adrenalectomized rats (*experiment 45*) daily with 0.5 ml. of whole extract (Parke, Davis and Co.) for one week and with 0.25 ml. for the second week we obtained values corresponding to those obtained with the control animals. One group (*experiment 51*) with intact adrenals was given a larger dose of whole extract, 2.0 ml. per day, and formed the same amount of new liver protein as the adrenalectomized group on the smaller dose. They reflected the increased cortical activity by having a more negative nitrogen balance than observed with the controls (*experiment 19*).

Merck's synthetic 11-dehydrocorticosterone acetate in oil was given subcutaneously in doses of 1 mg. per day. The animals (*experiment 60*) formed the same amount of new liver protein as did the controls (*experiment 19*) when fed the non-protein diet. When the rats (*experiment 59*) received casein in the postoperative diet the protein regeneration exceeded that of the controls (*experiment 14*), but was somewhat less than that observed with DCA treatment.

The effects of administering 17-hydroxy, 11-dehydrocorticosterone, in daily doses of 1 mg. was investigated in *experiments 61, 62 and 64*. Both the natural compound and the synthetic acetate were used. These animals did not do as well as other treated or control groups. Liver protein regeneration was essentially the same in all groups and approximated that of untreated controls with intact adrenals. While the nitrogen intakes were similar, the nitrogen balances varied from 0 to +.23 gm. This variation was not observed with other groups, and could not be correlated with clinical observations of the animals.

The composition of livers of the treated animals was not altered greatly from the normal values. However, animals treated with whole extract did have a somewhat higher liver glycogen value. The serum chlorides were lower in the DCA-treated rats, probably due to hemodilution because the DCA-treated group on plain water with the reduced intake had higher serum chloride values.

Histologically the liver, kidney, testes and thymus of the adrenalectomized animals were essentially the same as in the control animals. The perirenal fat tissue showed only an occasional collection of a few aberrant adrenal cortical cells. Grossly the thymus glands of the animals treated with adrenal cortical extract and DCA appeared smaller, and in untreated adrenalectomized rats they appeared somewhat larger than normally.

#### DISCUSSION

The observation that adrenalectomy depresses the regenerative power of the liver following its partial ablation has been described previously (10, 11). The decreased metabolism induced by removal of the adrenal glands probably accounts for some of the inhibition of new tissue formation. However, the present experiments suggest that the adrenal hormones might also be involved in protein synthesis. Ingle (12) states that under certain conditions adrenal cortical hormones favor anabolic

processes. He has shown that adrenalectomized, tube-fed animals do not gain weight as rapidly as intact animals or adrenalectomized animals treated with cortin.

Gurd, Vars and Ravdin (6) have demonstrated that the protein-depleted, partially hepatectomized rat will regenerate new liver protein even when fed a non-protein diet postoperatively. This is accomplished by the breakdown and mobilization of some endogenous protein and its resynthesis in the liver. The role of the adrenal cortical hormones in protein degradation has long been recognized. White and Dougherty (13) claim that the rate of mobilization of nitrogen from body tissues is influenced by the adrenal cortical steroids and thyroid hormone. The adrenal gland regulates the release of nitrogen from lymphoid tissue and the thyroid controls the rate of loss of nitrogen from the carcass. The utilization of protein-catabolites for protein regeneration is greatly aided by the adrenal corticoids (14). We were able to replace the impaired functions of the adrenalectomized rats by the administration of adrenal cortical extract as observed in the extract-treated groups. This is in accord with the observations of Berman *et al.* (10).

The effect of DCA on new liver protein formation is interesting in that it is not supposed to influence protein metabolism. That action of the adrenal cortex is thought to be maintained by the  $C_{11}$ -oxygenated steroids (15). Berman and her associates (10) reported an increase in liver weight of rats following DCA administration, especially when treated for 5 or 10 days. However, in animals treated for only 24 hours following partial hepatectomy they were unable to restore the normal amount of protein deposition as observed in their control animals. They state that this might be due to the inability of tissue to respond rapidly to DCA administered in oil.

Cowie and Foley (16) have found that DCA is more effective in maintaining lactation in adrenalectomized rats than the  $C_{11}$ -oxysteroids or whole adrenal cortical extract. This was accomplished without supplementing the drinking water with salt. Nagareda and Gaunt (17) believe that the improved lactation observed by Cowie and Foley with DCA was due to an improvement of the electrolyte balance of the adrenalectomized rats.

The somewhat increased water content of the livers and lower serum chloride values of the DCA animals are probably due to water retention and hemodilution observed with DCA therapy (18).

Our finding that 11-dehydrocorticosterone is more potent than 17-hydroxy, 11-dehydrocorticosterone is in accord with previous assays on the potencies of the steroids on growth and life maintenance (19, 20).

Much work remains to be done in studying the effects of the cortical steroids on protein metabolism. It is known that some breakdown of tissue proteins and resynthesis takes place continuously, but where the adrenal hormones fit into the cycle has not been proven. We have used the protein-depleted, partially hepatectomized rat as a means of investigating the action of the adrenal cortex on protein metabolism.

#### SUMMARY

Removal of the adrenal glands impaired the deposition of protein in the liver following partial hepatectomy. In the adrenalectomized rat administration of ad-

renal cortical extract restored the liver's regenerative power to form new tissue protein. Desoxycorticosterone acetate stimulated the liver to synthesize even more new liver protein following partial hepatectomy than occurred in animals with intact adrenal glands. 11-Dehydrocorticosterone acetate was more potent than 17-hydroxy, 11-dehydrocorticosterone and almost equal to DCA in restoring the ability of adrenalectomized rats to regenerate new liver protein.

We are grateful to the following for generously supplying us with the hormone products used in this investigation: Dr. Hans Molitor of Merck and Company for 11-dehydrocorticosterone acetate and 17-hydroxy, 11-dehydrocorticosterone acetate; Dr. F. F. Yonkman of Ciba Pharmaceutical Products, Inc. for desoxycorticosterone acetate; Dr. J. J. Pfiffner of Parke, Davis and Company for 17-hydroxy, 11-dehydrocorticosterone and whole adrenal cortical extract; and Dr. D. J. Ingle of the Upjohn Company for whole adrenal cortical extract.

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# IDENTITY AND SPECIFICITY OF FROG WATER-BALANCE PRINCIPLE OF POSTERIOR PITUITARY EXTRACT

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THE injection of posterior pituitary extract causes a striking increase in the body water content of frogs kept in water. Brunn (1) first described this water-balance effect and found that it did not depend on the suppression of urine output since it was not eliminated by cloacal ligation or nephrectomy. These observations have been confirmed by many authors working with many species of amphibians (2).

A simple assay method for the water-balance principle using *Rana pipiens* has been applied to the investigation of the relative water-balance activity present in whole posterior pituitary extract and in the oxytocic and pressor fractions. Intermedin and several steroid hormones have also been investigated for water-balance activity in order to test the specificity of this response.

## METHODS

Male *Rana pipiens* obtained from a Vermont supplier, averaging about 30 gm. in weight were used. They were kept in 100 cc. of tap water in individual beakers at room temperature for at least 16 hours before an experiment and during each experiment. Under these circumstances weight fluctuations of the control animals were usually slight.

At the start of each experiment urine was expressed by abdominal pressure, the frog carefully dried in a towel and weighed in a light, covered plastic dish on a torsion balance to within 0.1 gm. This procedure was followed for all subsequent weighings. Cloacal ligation was accomplished by tying a cotton thread about the aperture with a single bowknot to facilitate removal.

Aqueous solutions were injected into the dorsal lymph sac, oily solutions intraperitoneally. Controls received water, chlorotone, phenol, sodium chloride and sesame oil in amounts corresponding to the quantities of these substances present in the experimental injections.

In most of the experiments reported a crossover technique was used. Two groups of frogs were used simultaneously. The first group received the extract, the second served as controls. One or two days later these groups were reversed and the experiment repeated. Thus each group served as its own control.

Numerical results are expressed as arithmetic means plus or minus the standard errors of the means. Differences between means and the standard errors of the differences are used to determine statistical significance. *P* values are from Fisher's (3) table of *t* for small samples. A result is considered significant if *P* is less than 0.01. Points plotted in the figures represent arithmetic means, vertical lines through points indicate the standard errors.

## RESULTS

*Relationship of Water-balance Effect to Dose of Posterior Pituitary Extract.* Whole posterior pituitary extract (Pituitrin, Parke, Davis & Company) was given to frogs

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in 330 individual experiments in doses ranging from 0.063 to 8.0 units/100 gm. body weight. All injections were made in a standard volume of one cc/100 gm. and contained 0.5 per cent chlorethone as a preservative. One cc. of 0.5 per cent chlorethone was given to all control frogs. Such a dose of chlorethone was without effect on water balance when compared to distilled water in experiments on 20 additional frogs.

The maximal weight change in frogs injected in this manner occurred at  $2\frac{1}{2}$  to  $3\frac{1}{2}$  hours after injection. The endpoint chosen for evaluation of the response was therefore the weight gain in 3 hours. The lower curve in figure 1 represents the 3-hour weight gain in percentage of initial body weight plotted against the dose of pituitrin on a logarithmic scale. There is great seasonal variation in the response of frogs to the water-balance principle and these figures represent frogs used in the early summer only.

The minimal effective dose of pituitrin appears to be about 0.05 units/100 gm. A straight regression line can be drawn from this dose level to a level of approximately one unit. At this point the slope decreases only to increase again between 3 and 4 units. At doses above 4 units there is no further increase in response. The shape of the weight-gain curve suggests that two mechanisms may be involved and that this is in reality a compound dose-response curve.

An additional series of experiments was undertaken to try to explain the second upward deflection of the weight-gain curve. In 65 individual experiments frogs were given varying doses of pituitrin after cloacal ligation. Since ligation often caused necrosis of the skin about the cloacal aperture this procedure could not be repeated on the same frogs. Therefore 31 separate control experiments were carried out simultaneously.

Control frogs with ligated cloacas gained  $4.6 \pm 0.5$  per cent in 3 hours. It is assumed that this represents the normal rate of water uptake and urine formation in frogs under these circumstances. That cloacal ligation does not suppress urine formation was shown by the fact that these frogs returned to their initial weights immediately after the ligature was removed and the urine expressed from the bladder. The upper curve in figure 1 represents the water uptake of the control frogs and those given pituitrin. Since urine output has been prevented by cloacal ligation the differences between the weight gain of these frogs given pituitrin and the controls cannot represent differences in urine output. The extra weight gain must represent increased water uptake. Since frogs do not drink water (4) this apparently represents an increased rate of water intake through the skin.

The curve for water uptake resolves into a simple dose-response curve with a maximal response occurring at about 1 unit/100 gm. The secondary rise in the weight-gain curve must represent a decrease in urine output in frogs given a large dose of pituitrin. The mechanism of this effect will be the subject of a subsequent paper.

*Comparative Water-balance Activity of Oxytocic and Pressor Fractions of Posterior Pituitary Extract.* Varying doses of oxytocic (Pitocin, Parke, Davis & Company) and pressor (Pitressin, Parke, Davis & Company) fractions of posterior pituitary extract were given to 90 frogs. It appears that pitocin has water-balance activity essentially equal to that of pituitrin (fig. 2). Pitressin has approximately one-tenth the water-balance activity of pitocin or pituitrin. It is probable that the water-

balance activity of pitressin represents that of the small amount of oxytocic principle present (2).

*Effect of Intermedin on Water Balance in the Frog.* Pitocin contained approximately ten times the frog water-balance activity present in pitressin. Pitressin, however, appeared in the previous experiments to contain more melanophore-expanding activity than pitocin. This had also been noted by Rowe (5) and Gaddum (6). More specific experiments were undertaken to determine whether or not the frog water-balance principle could be completely dissociated from melanophore-expanding principles.

Treatment with alkalis rapidly destroys posterior pituitary principles. The intermediate lobe hormone, intermedin, is stable for several hours in alkali at room temperature (7). Pitocin was treated with 1N NaOH for 3 hours at room temperature,

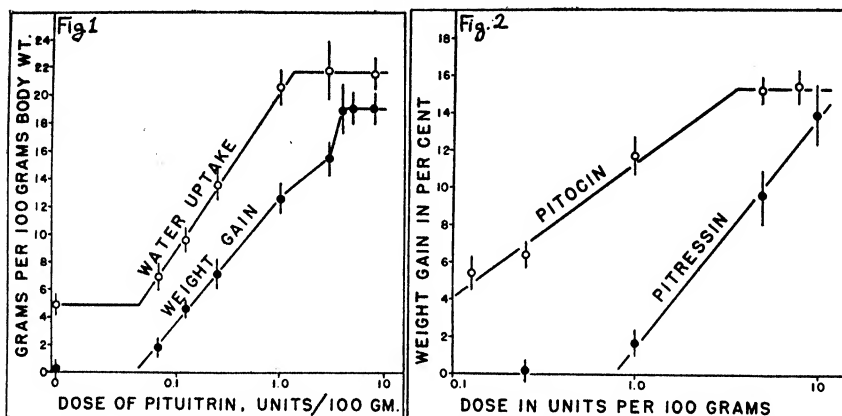


Fig. 1. RELATIONSHIP BETWEEN DOSE OF PITUITRIN AND WEIGHT GAIN AND WATER UPTAKE by frogs in 3 hours following injection.

Fig. 2. RELATIONSHIP BETWEEN DOSE OF PITOCIN OR PITRESSIN AND WEIGHT GAIN by frogs in 3 hours following injection.

neutralized with HCl and injected into a series of frogs. One control group received untreated pitocin to which NaCl had been added in the same concentration and another received just NaCl. It is evident that the water-balance activity of pitocin was completely destroyed by such alkali treatment (table 1).

These experiments were repeated using a concentrated preparation of sheep intermedin supplied by Dr. E. H. Frieden of this laboratory. This extract was known to be stable in 1N NaOH at room temperature. We administered a dose of 2 mg. of powder/100 gm. body weight. This corresponded to the melanophore-expanding activity of approximately 3 mg. (6 units) of International Standard Posterior Pituitary Powder. The alkali completely destroyed the water-balance activity of this extract (table 2). The alkali-treated extract did, however, contain enough melanophore-expanding activity to prevent the frogs from light-adapting under a strong light against a white background for approximately 48 hours. This would appear to be strong evidence against the identity of water-balance and melanophore-expanding principles in pituitary extracts.

*Effect of Steroid Hormones on Water Balance in the Frog.* The body weights of individual frogs in groups of 12 to 16 were followed at intervals for 24 hours after the injection of each of the following steroid hormones: Desoxycorticosterone acetate, 0.5, 1.0, 2.0 and 5.0 mg/100 gm., desoxycorticosterone glucoside, 1.0 mg/100 gm., adrenal cortical extract (Eschatin, Parke, Davis & Company) one cc/100 gm., adrenocorticotrophic hormone (Armour), 1.0 mg/100 gm., estradiol, one mg/100 gm., progesterone, one mg/100 gm., and testosterone propionate, 10 mg/100 gm. None of these produced weight changes differing significantly from the controls, even at these relatively high doses.

## DISCUSSION

Many workers (8-11) have found that oxytocic extracts are more effective than pressor extracts in increasing the water uptake of frogs. Novelli (12), however, reported that pitressin was more effective than pitocin in causing water uptake in the toad *Bufo arenarum*. We have developed an assay technique using *R. pipiens* that has made a more quantitative approach to this problem possible. The dose-

TABLE 1. EFFECT OF ALKALI TREATMENT ON WATER BALANCE ACTIVITY OF PITOCIN<sup>1</sup>

EXTRACT	ALKALI-TREATED PITOCIN		UNTREATED PITOCIN		DIFFERENCE Untreated - Treated
	No.	Wt. change	No.	Wt. change	
Dose					
1 unit/100 gm.	8	-0.4 ± 1.2	6	10.3 ± 1.6	11.7 ± 2.6 ( <i>P</i> < 0.01)
2.5 units/100 gm.	10	0.7 ± 1.7	10	11.1 ± 2.6	10.4 ± 2.4 ( <i>P</i> < 0.01)

<sup>1</sup> Values shown represent difference between the average weight of injected frogs and control frogs at 3 hours, plus or minus the standard error of the differences, in percentage of initial body weight.

response curve for pitocin falls almost exactly on the pituitrin curve. Pitressin has approximately one-tenth the activity of pitocin or pituitrin. This would bear out Heller's (2) suggestion that the oxytocic fraction contains the water-balance principle and that the water-balance activity of pressor extracts is due to the presence of oxytocic principles in these extracts.

The water-balance principle may not be identical with the oxytocic principle. Heller (13) has shown that the amounts of water-balance and oxytocic activities in neurohypophyseal extracts from various vertebrate classes vary quite independently. It must be remembered, however, that these assays by their nature cannot be carried out on the same animals and that, since these hormones are protein-like in nature, species and class specificities may make such comparisons misleading. Heller and Smith (14) have also shown that although crustacean eye-stalk extracts contain frog water-balance activity they have no oxytocic activity.

The specificity of posterior lobe extract in producing the amphibian water-balance effect has been indicated by the failure of Brunn (1), Biassoti (15), Heller (16, 13), and Boyd and Gibson (17) to demonstrate any significant water-balance effect in extracts of the anterior lobe of the pituitary. Most posterior lobe extracts contain melanophore-expanding activity, however. That this hormone is not responsible for the water-balance effect was indicated by Heller (13) who found that pigeon



anterior lobe extract contained no frog water-balance activity although it contained the melanophore-expanding hormone. Oldham (10) also observed that a potent melanophore-expanding extract did not affect the rate of elimination of saline by frogs. The excretion of injected saline, however, probably has no direct bearing on the true water-balance effect.

Brunn (1) found no water-balance activity in ovarian extracts. Biassoti (15) found adrenal cortical extracts to be without water-balance effect in the frog. Dow and Zuckerman (18) injected high doses of several steroids into axolotls. They found that estrone, progesterone and testosterone propionate were effective in producing a gain in weight of axolotls kept in water. They also observed a slight but statistically significant fall in body weight after the injection of desoxycorticosterone acetate or adrenal cortical extract. Hsieh (19), however, observed no significant weight change in *R. pipiens* given one mg. desoxycorticosterone acetate or one cc. of adrenal cortical extract.

It was found that melanophore-expanding activity and water-balance activity of pituitary extracts can be easily separated by treatment with alkali. Thus melano-

TABLE 2. EFFECT OF ALKALI TREATMENT ON WATER-BALANCE ACTIVITY OF AN INTERMEDIN PREPARATION

EXTRACT	NO.	3-HOUR WEIGHT GAIN	DIFFERENCE FROM CONTROLS
Alkali-treated intermedin	14	$2.1 \pm 0.5$	$0.4 \pm 0.8$ ( $P, 0.6-0.7$ )
Untreated intermedin	15	$9.3 \pm 1.6$	$7.6 \pm 1.7$ ( $P < 0.01$ )
Controls (NaCl)	12	$1.7 \pm 0.6$	
Difference, untreated minus treated extract			$7.2 \pm 1.7$ ( $P < 0.01$ )

phore-expanding hormone of the intermediate lobe of the pituitary cannot be responsible for the water-balance effect of posterior pituitary extracts. The injection of steroid hormones did not produce significant changes in water balance. The doses used were, however, somewhat lower than those used by Dow and Zuckerman (18) in the axolotl. This fact, and species differences, probably explain the failure to observe similar changes in the frog.

#### SUMMARY

Posterior pituitary extracts influence the water balance of frogs by both renal and extrarenal mechanisms. At lower doses the extrarenal effect, consisting of an increased rate of water uptake, predominates. This response has been employed as a means of assay for water-balance activity. The minimal effective dose of pituitrin is approximately 0.05 units/100 gm. body weight. A maximum effect on water uptake is found at doses over one unit/100 gm. The water-balance principle of posterior pituitary extracts is contained predominantly in the oxytocic fraction. Pitocin contains the same activity as pituitrin and is ten times as active as pitressin.

A concentrated preparation of the melanophore-expanding hormone from sheep pituitaries had no significant effect on frog water balance. Desoxycorticosterone acetate, desoxycorticosterone glucoside, adrenocorticotrophic hormone, adrenal cor-

tical extract, estradiol, progesterone, and testosterone propionate exert no definite effect on frog water balance even in relatively high doses. The water-balance principle appears to be a specific property of the oxytocic fraction of posterior pituitary extract.

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# TOXICITY OF SEA WATER IN MAMMALS

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THE effects of administering sea water and similar solutions to animals, and occasionally to human beings, have been studied sporadically. Semipopular accounts of the gross effects of the drinking of sea water by shipwreck victims exist (1). During World War II the torpedoing of ships at sea and the loss of planes over open ocean, setting survivors adrift in lifeboats and on rafts, gave impetus to careful investigation of the question whether it is beneficial or harmful for castaways to drink sea water (2-5).

The present studies, on several species, were designed to elucidate the following questions: *a)* How much sea water can be tolerated by mammals, and by which routes of administration? *b)* What animals are most resistant to the toxic effects of sea water? *c)* What bodily effects do lethal and sublethal doses of sea water exert? *d)* How do excess salts and water distribute themselves? *e)* How may animals' resistances to sea water be modified, if at all? *f)* Can animals be induced to drink sea water? These questions bear not only upon the toxicity of sea water for men who are forced to drink it or nothing, but also upon physiological regulations of water and salt in mammals.

The composition of sea water might suggest the nature of its toxic effects: does sea water cause damage to men and animals simply by dehydrating them through its high osmotic concentration, or are one or more of its ions specifically toxic?

These studies included the use of several solutions: artificial sea water prepared approximately according to the formula of Harvey (6); sea water from Woods Hole, Massachusetts; water from the Gulf of Maine; and solutions of  $MgCl_2$ , of  $NaCl$ , of sucrose and of urea. The Woods Hole sea water is assumed to have had the composition reported for it by Page (7); our chloride analyses agreed very closely with his.

The salinity of sea water averages 3.4 per cent (0.58 molar), though it varies in different localities. The proportions of the diverse ions in sea water also vary somewhat, but the  $Cl^-$  ion is most abundant and the  $Na^+$  ion is next, and these two together far exceed all other ions combined (6). Concentrations of  $Na^+$  and  $Cl^-$  in sea waters are three to five times as great as the normal concentrations of these ions in human serum; of  $Mg^{++}$ , four times that serum concentration which causes death from cardiac arrest in mammals, and seven times that serum concentration which causes cessation of respiration;  $Ca^{++}$  and  $K^+$  in sea water are about two-thirds of the fatal serum  $Ca^{++}$  and  $K^+$  concentrations in mammals. All of the other ions in

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sea water are well below their toxic concentrations in serum (8, 10). Hence we investigated the toxicities of only  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{Mg}^{++}$  ions in sea water.

$\text{Na}^+$  and  $\text{Cl}^-$  are the predominant ions of the extracellular fluid, and their turn-overs in the animal body are great. Apparently their bodily effects are chiefly osmotic; changes in their concentrations in body fluids result in serious disturbances of fluid distribution (8).  $\text{Mg}^{++}$  in serum concentration of only 10 mm/l. has a specific toxicity for tissues, especially for the central nervous system and the heart (9, 10). It seems unlikely that the pH of sea water, which is between 7.9 and 8.3 (7), is in any degree responsible for its toxicity.

#### METHODS

Most of the experiments were made on albino rats. Guinea pigs, mice, dogs and seals were used to limited extents. For the most part the solutions being studied were given by intraperitoneal injection, the only route by which sea water itself can be given in a single quick dose large enough to be lethal. All solutions were given at 37° C. Doses of the various solutions given are expressed as 'per cent of body weight,' that is, ml/100 gm. of body weight.

$\text{Cl}^-$  analyses were done by the method of Schales, Schales and Asper (11, 12). Blood was obtained by cutting the tip of the tail in rats or by venipuncture in seals and dogs. Heparin was used as anticoagulant. Total solids concentration of supernatant plasma was measured by a dipping refractometer.

Most of the animals that died were autopsied, to distinguish deaths from trauma or from extraneous causes. Frequently animals which survived an experiment were used again one or more times, after ample recovery periods. In some experiments it was essential to use survivors for further tests.

#### EXPERIMENTS WITH RATS

*Artificial Sea Water.* To ascertain the lethal dose, a single intraperitoneal injection of 16 per cent of the body weight of artificial sea water (0.456 M  $\text{Cl}^-$ ) was given. It was lethal for 5 of 9 adult male rats, and all that died had convulsions. Tests with females failed to show a sex difference here. In half of 8 young rats of 30 to 90 gm., 17 per cent of the body weight was lethal. No convulsions occurred in any of the young.

*Natural Sea Water.* a) *By intraperitoneal injection.* Woods Hole sea water in doses of 12 per cent of the body weight was non-lethal for 10 adult male rats, but in doses of 14 or 16 per cent was fatal to 21 of 27 animals (78%). The incidence of convulsions in rats that died was much less than in those which succumbed to artificial sea water. The higher concentration of  $\text{Mg}^{++}$  in the natural sea water may have exerted a depressant effect upon the central nervous system.

In table 1, rats given intraperitoneal injections of artificial sea water, natural sea water, or 0.5 M NaCl solution are included together in group I, as there were no statistically significant differences among the mortalities from these solutions.

The experiments reported above were carried out on rats in presumably optimal states of nutrition and hydration. Laboratory temperature varied little from 24° C. Following test injections the animals were denied food and water for 8 hours and were kept in individual, wire, funnel-bottom cages for collection of urine.

Since human beings who resort to drinking sea water are usually those who have been subjected to dehydration with or without starvation, the influence of lack of

body water on the toxicity of sea water was tested. Twelve male rats were deprived of water for 1, 2 or 3 days before injection of a 12 per cent dose of natural sea water. Nine animals died, within 2 to 96 hours, all with convulsions (table 1, group Ib). The mortality was directly proportional to the number of days of dehydration. The high incidence of convulsions may have meant that in dehydrated animals the 12 per cent dose of sea water killed by its osmotic effects without building up a sufficiently high  $Mg^{++}$  concentration in body fluids and tissues to have an anticonvulsant effect.

TABLE 1. MORTALITIES AND INCIDENCE OF CONVULSIONS IN ANIMALS GIVEN SEA WATER OR OTHER SOLUTIONS BY STOMACH TUBE (S.T.) OR BY INTRAPERITONEAL INJECTION (I.P.)

GROUP	ROUTE OF ADMIN.	SOLUTION GIVEN	DOSE, % OF BODY WT.	SPECIES	NO. OF ANIMALS	NO. OF DEATHS	NO. WHICH HAD CONVULSIONS	TIME TO DEATHS
								hours
Ia	i.p.	Sea water or 0.51 M NaCl	14-16	Rat	56	38	14	2 - 240
b	i.p.	Sea water after water deprivation	12	Rat	12	9	9	2 - 96
II	s.t.	Sea water after deprivation	12	Rat	18	3	1	10 - 39
IIIa	i.p.	1.0 M sucrose	14-18	Rat	11	11	10	1.7 - 2.0
b			14	Mouse	8	8	0	1 - 20
c			6-7	Guinea pig	4	4	4	3 - 8
IVa	i.p.	$MgCl_2 + CaCl_2$	16	Rat, young	18	3	0	3 - 8
b			16	Rat, old	11	10	0	2 - 23
Va	i.p.	Sea water	16	Mouse	17	11	0	1 - 48
b			16	Guinea pig	6	5	0	8 - 25
VI	i.p.	Sea water + R	16	Rat	11	3	0	1.1 - 1.7

b) *By stomach tube.* Table 1 (group II) shows the mortality in rats given three 4 per cent doses of sea water by stomach tube 40 minutes apart, following 3 or 4 days' deprivation of food and water or of food alone. Deprivation of food alone had the same effect as total deprivation, since the starving animals drank very little. Rate of weight loss in all groups was markedly accelerated by the oral administration of sublethal amounts of sea water.

Only one of our rats was known to have convulsions after oral administration of hypertonic NaCl solutions, but Ulrich and Shternov (13) reported convulsions in rats and other species following oral as well as intraperitoneal administration of NaCl solution.

*NaCl Solution Isotonic with Sea Water.* Solutions of pure NaCl were tested intraperitoneally in an attempt to learn whether or not lack of  $Mg^{++}$  and other lesser

constituents of sea water make a difference. The mortality from pure NaCl solutions is like that from natural sea water, though the incidence of convulsions in animals that died from NaCl is greater. Perhaps the difference is again due to the depressant effect of  $Mg^{++}$  upon the central nervous system.

*Concentrated Artificial Sea Water and Concentrated NaCl Solution.* How fast could fatal shifts of body fluids and electrolytes be produced in rats by giving salt solutions several times more concentrated than sea water? Artificial sea water was evaporated to a  $Cl^-$  concentration of 1.05 M, or 2.2 times the original, and 0.51 M NaCl solution to a  $Cl^-$  concentration of 1.10 M. These concentrations were arbitrarily chosen.

The volumes of either concentrated solution that were administered contained the same amount of salt as a 15 per cent dose of sea water. All 7 rats died in  $\frac{1}{2}$  to 4 hours without convulsions, whether the salts were received by intraperitoneal injection or by stomach tube. Hence, with less water, the same amounts of salts were more lethal, as might be expected from the higher final concentration then attained within certain body fluids. It is also noteworthy that rats showed no convulsions when they lived less than 2 to 4 hours after solutions had been given.

*Sucrose Solutions (1.0 M) Isotonic with Natural Sea Water.* It seemed that the toxic effects of sea water might be due simply to the dehydrating effect of its high salt content. Could similar effects be produced in rats by dehydrating them with a solution of a non-electrolyte such as sucrose? With 14 to 18 per cent doses of intraperitoneal sucrose solution all of the rats died (table 1, group III); hence, we do not know the exact toxic dose of 1.0 M sucrose solution in this species. Since sucrose is digested in the alimentary tract, oral solutions of it cannot be compared with sea water by that route.

*Concentrated Urea Solutions.*<sup>2</sup> The effects of a concentrated solution of urea, a solute which moves freely across cell membranes and distributes itself throughout most of the body water (14, 15), were compared with the effects of a concentrated solution of NaCl, a solute presumably confined almost wholly to extracellular spaces (16).

Of 2 groups of 6 rats each, one group was injected intraperitoneally with 4 per cent of the body weight of 1.65 M NaCl solution, the other group with the same dose of 3.0 M urea in isotonic (0.15 M) or slightly hypotonic (0.10 M) NaCl solution. Hemoglobinuria occurred in some rats injected intraperitoneally with urea solution; and the  $Cl^-$  concentration of the peritoneal fluid changed to that of the serum (0.10 M).

All 12 animals died (table 2). The rats given concentrated NaCl solution had no convulsions, but became limp; those given concentrated urea solution had convulsions, which consisted of marked shivering followed by tonic and clonic movements, hurling the body around the cage.

*Salt Solutions Isotonic with Plasma.* The question naturally arose whether or not part of the toxicity of intraperitoneal sea water might not be due to the mechanical effects of the injected solution. Adult rats were given single, graded doses of

<sup>2</sup> I am indebted to Adelaide D. Brokaw for these experiments. The data are abstracted from a thesis presented by her in partial fulfillment of the requirements for the degree, Master of Science.

0.154 M NaCl solution or of natural sea water diluted to be isotonic with plasma; 10 rats were used on 16 different occasions. It was found that 52 per cent of the body weight of either solution could be injected into the animals' peritoneal cavities with impunity. That volume of diluted sea water contained the same amount of salt as a 16 per cent dose of the undiluted sea water. (It required 22 to 37 hours for the animals to return to their original body weights, despite deprivation of food and water during that time.) Thus it seems that the concentration of salt given, as well as the total amount, can be important in the toxicity of intraperitoneal hypertonic salt solutions.

*Role of  $Mg^{++}$  in the Toxicity of Sea Water.* In 2 adult male rats given intraperitoneal doses of 16 per cent of the body weight of  $MgCl_2$  solution containing  $Mg^{++}$  in the same concentrations as in sea water, convulsions and death resulted in 17 minutes. The depressing effects reported for  $Mg^{++}$  in lesser doses were not here observed.

Table 1 (group IV) shows the results of the intraperitoneal administration to rats of a solution containing  $MgCl_2$  and  $CaCl_2$  as in sea water. Group IVa consisted

TABLE 2. COMPARISON OF EFFECTS OF INTRAPERITONEAL INJECTIONS OF 1.65 M NaCl AND OF 3. M UREA IN RATS. MEANS AND STANDARD ERRORS ARE SHOWN

		NaCl	Urea
Survival time (minutes)		33±2	31±1
Increase in peritoneal fluid volume (% of body weight)		2.4±0.4	1.4±0.1
Estimated change in plasma volume (% of initial volume)	From hematocrit ratio	+49±10	-33±12
	From plasma solids concentration	+16±5	-23±5
Convulsions		None	All

of young adult males and females, group IVb of all old males; the high mortality in the latter group may have been due to age. There were no convulsions from these solutions. The data for group IVa suggest that  $Mg^{++}$  is effectively antagonized by  $Ca^{++}$ , as is generally accepted.

Rabbeno (17) reported that lethal doses of intravenous sea water in guinea pigs caused predominant symptoms of depression, similar to those caused by  $Mg^{++}$ . He (18) also reported that sea water injected into veins of rabbits was more toxic than a NaCl solution, and believed this greater toxicity of the former to be due to its  $Mg^{++}$  content with (relative)  $Ca^{++}$  deficiency. We find no evidence that this is so in rats.

The role of  $Mg^{++}$  in the toxicity of *ingested* sea water is certainly nonspecific. Magnesium salts are little absorbed from the gastrointestinal tract of mammals and rarely reach toxic levels in the blood unless excretion of  $Mg^{++}$  is poor. The salts are evacuated from the gut in isotonic solution and have a dehydrating effect if given in hypertonic solution (9).

Two rats were given, by stomach tube, doses of 1 and 2 per cent of the body weight of hypertonic  $MgCl_2$  solution (0.64 M) with no ill effects. The same doses given intraperitoneally killed 2 other rats, after convulsions, within 10 minutes. Thus the  $Mg^{++}$  ion probably contributes nothing specific to the toxicity of ingested sea water, though it may modify the effects of parenteral sea water, especially by the intravenous route.

## CHANGES IN BODY FLUIDS

**Salt Solutions.** Injection into a rat's peritoneal cavity of a sea water or NaCl solution having almost four times the osmotic pressure of plasma results in an influx of body fluid into the peritoneal cavity, simultaneous with  $\text{Cl}^-$  absorption (increasing the plasma  $\text{Cl}^-$  concentration), both processes occurring most rapidly immediately after injection.

Table 3 shows changes of peritoneal fluid volume and dehydration in rats dying from or killed after injection of sea water. Obviously at some point passage of fluid into the peritoneal cavity ceases and resorption begins. Animals dying several hours after injection invariably had plasma  $\text{Cl}^-$  concentrations between 175 and 200 mEq/l. In a single case in which a rat was injected intraperitoneally with 43 per cent of the body weight of a 1.1 M NaCl solution, death occurred 15 minutes after the injection and the terminal plasma  $\text{Cl}^-$  concentration was 244 mEq/l. If a rat survived at least 30 hours after an injection, peritoneal fluid was completely absorbed and plasma  $\text{Cl}^-$  concentration was often slightly below normal. Cunningham (19) found that intraperitoneal injections of 10 per cent dextrose solution in rats increased the peri-

TABLE 3. DEHYDRATION AND PERITONEAL FLUID VOLUMES IN ADULT MALE RATS GIVEN INTRA-PERITONEAL INJECTIONS

SOLUTION GIVEN, 16% OF BODY WT.	NO. OF RATS	NO. WHICH HAD CONVULSIONS	HOURS TO DEATH, MEAN	DEHYDRATION, % OF WT.	CHANGE IN PERITONEAL FLUID
					vol. %
1.0 M Sucrose	Died 8	5	1.7	12.8	+69
Sea water	Died 22	0	2.1	6.7	+13
Sea water	Killed { 3	2	10.5	9.9	-17
	4	1	23.3	10.5	-65

toneal fluid volume, which reached a maximum in 4 hours and thereafter decreased until fully absorbed in 12 to 13 hours. We found that animals killed by intraperitoneal hypertonic salt solutions were much more dehydrated at death than would be expected on the basis of only renal and evaporative water losses at control rates during the time since injection. Dehydration was calculated in terms of per cent loss of body weight; rats were weighed before injection of sea water, and at death after removal of peritoneal fluid and bladder urine. A high plasma  $\text{Cl}^-$  concentration was associated with a decreased hematocrit ratio (fig. 1, table 2). This is believed to indicate an increase in extracellular fluid volume accompanying the  $\text{Cl}^-$  excess.

Table 4 shows calculations, by two different methods, of extracellular and intracellular fluid volumes at death in rats given intraperitoneal injections of 16 per cent of the body weight of sea water. The data are means of values obtained in 13 rats. The values for the volumes of distribution of  $\text{Cl}^-$  calculated by two methods are identical. The increase in extracellular fluid is at the expense of intracellular volume in animals dead several hours after intraperitoneal administration of sea water. Intraperitoneal injections of 0.85 M NaCl solutions in dogs (5) suggest the same conclusions. Cellular dehydration may be sufficient to produce both convulsions and death from sea water and similar solutions. It seems likely that the central



nervous system is the first to be affected. Winkler *et al.* (5) could find no evidence, nor could we, that circulatory failure precipitated death. The type of death observed in our animals, with or without convulsions, may depend upon the site and nature of damage to the central nervous system.

**Sucrose Solution.** Following the intraperitoneal injection of molar sucrose solution in rats there is an even greater influx of body fluid into the peritoneal cavity, possibly because sucrose molecules are moving only slowly across the peritoneal membrane. At death these animals showed greater dehydration than did rats given sea water and dying in the same length of time (table 3). Also, they showed increased hematocrit ratios, indicative of hemoconcentration. Hence in these animals both intracellular and extracellular fluid compartments dwindled, and the lost fluid was all in the peritoneal cavity (practically none was excreted). Convulsions and death

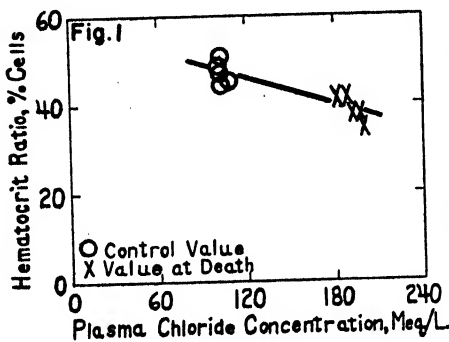
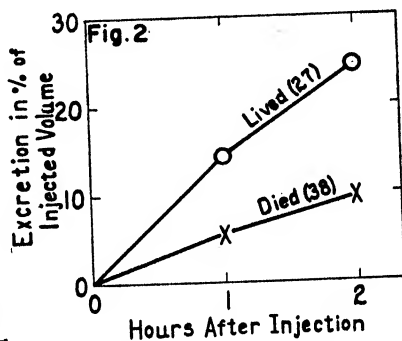


Fig. 1. RELATION BETWEEN HEMATOCRIT RATIOS AND PLASMA CHLORIDE CONCENTRATIONS in rats given intraperitoneal injections of 16 per cent of body weight of sea water or 0.5 M NaCl solution.

Fig. 2. URINE EXCRETED BY SURVIVING AND NON-SURVIVING RATS after intraperitoneal injection of 14 to 18 per cent of body weight of sea water or 0.5 M NaCl.



from sucrose solution may be due to both cellular and extracellular dehydration; in the case of sea water they may result from cellular dehydration alone.

**Urea Solution.** At death from the 3.0 M urea solutions injected (above), samples of blood and peritoneal fluid were removed and analyzed (table 2). The volume of peritoneal fluid increased, but to a less extent than at death from isosmotic NaCl solutions. The urea led to decrease of estimated plasma volumes. If we assume that this decrease reflects a corresponding decrease in extracellular fluid exclusive of peritoneal fluid, then the conclusion is that water has entered cells, because the increase in peritoneal fluid is too small to account for the calculated decrease in total extracellular fluid exclusive of that in the peritoneal cavity. In the animals given urea, convulsions and death occurred in the presence of decreased plasma and extracellular fluid volumes and of presumably increased cellular water, the reverse of the situation obtaining with NaCl.

**Comparisons.** Electrolyte disturbances involving more than just the  $\text{Na}^+$  and  $\text{Cl}^-$  ions may contribute to the deaths of the animals given excess salt. Schilling (20) states that excess  $\text{Na}^+$  drives  $\text{K}^+$  out of cells into the extracellular fluid. Elkin-ton *et al.* (21) say that exchanges of  $\text{Na}^+$  for  $\text{K}^+$  in cells do occur, but  $\text{K}^+$  leaves cells

in significant amounts only if it can be excreted. It may be noted here that many of our rats died anuric, especially after being given the highly concentrated NaCl solutions. A marked increase in extracellular  $K^+$  is injurious to cells, and injured cells lose  $K^+$ , constituting a vicious cycle if plasma  $K^+$  is allowed to rise (22). These authors believe that it is difficult to disturb the  $K^+$  regulating mechanism of a healthy animal; but obviously animals intoxicated with sea water and anuric cannot be considered healthy. Normal mice poisoned with  $K^+$  die with convulsions (23), and we found that 2 rats given 12 per cent of the body weight of 0.5 M KCl solution

TABLE 4. CALCULATIONS OF MEAN EXTRACELLULAR AND INTRACELLULAR FLUID VOLUMES IN 13 RATS AT DEATH FROM INTRAPERITONEAL SEA WATER

	ml. of mEq/kg. Rat	mEq/l. %
a. Dose	160	
b. $Cl^-$ conc. administered		500
c. Normal volume extracellular fluid, Harrison <i>et al.</i> (16)	290	
d. Normal volume intracellular fluid, (16)	380	
<i>Method A</i>		
e. Normal plasma $Cl^-$ conc., determined		103
f. Total body $Cl^-$ before sea water, (16)	30	
g. Peritoneal fluid vol. at death, determined	178	
h. Peritoneal fluid $Cl^-$ conc. at death, determined		229
i. $Cl^-$ absorbed from peritoneal fluid, $(a \times b) - (g \times h)$	39	
j. Total body $Cl^-$ (minus perit. fluid) at death, $(f + i)$	69	
k. Plasma $Cl^-$ conc. at death, determined		192
l. Vol. of distribution of $Cl^-$ (= extra-cell. vol.) at death, $(j/k)$	60	
m. Total body fluid lost at death, determined	51	5.1
n. Intracellular fluid vol. at death $(c + d - m - l)$	260	
o. Decrease in intracellular fluid at death, $(d - n)$	120	32
<i>Method B</i>		
p. Normal hematocrit ratio, determined		47.3
q. Hematocrit ratio at death, determined		34.0
r. Extracellular fluid vol. at death $c(100 - q)/(100 - p)$	360	
s. Increase in extracellular fluid vol. at death, $(r - c)$	70	24
t. Intracellular fluid vol. at death, $(d - s - m)$	260	
u. Decrease in intracellular fluid at death, $(d - t)$	120	32

intraperitoneally died within 12 and 29 minutes with cyanosis and asphyxial convulsions. These convulsions were not, however, considered similar to those occurring from sea water or NaCl solution.

The experiments with urea clearly suggest that shrinkage of cellular compartments is not necessary to the lethal effects of solutions. If sea water kills by cellular dehydration, urea solutions kill otherwise. It is unlikely that urea has a specific toxic effect. At present the osmotic theory of death requires more detailed modifications before it can fit all the data.

#### ADAPTATION IN TOLERANCE

*Lethal Doses of Hypertonic Salt Solutions.* Could rats be rendered resistant to ordinarily lethal doses of sea water or similar solutions by repeated sublethal doses? The 'adaptation' procedures used (table 5) varied slightly. The longest period of

sublethal injections consisted of 15 intraperitoneal doses within 18 days. The first 'adapting' dose given was usually 10 per cent of the body weight; all others were 12 per cent. One group of control animals was given 0.154 M NaCl solution; another was given only sham injections. Then all groups were tested with a 16 per cent dose of sea water or hypertonic NaCl solution. Adaptation was clearly demonstrated in those animals previously given sublethal injections. 'Adapted' individuals that survived the ordinarily lethal test dose were retested with the same dose after a period of 'deadaptation.' Six to 8 hours after each test dose, surviving animals again had food and water *ad libitum*.

Table 5 shows that after a period of 'deadaptation,' which varied from 3 to 21 days after the first test dose was given, most of the experimental survivors succumbed to the 16 per cent retest dose of a hypertonic salt solution as readily as the unadapted controls had previously. Thus the adaptation is a transient phenomenon, whatever its nature; it can be produced in as few as 3 days and disappears with equal rapidity.

TABLE 5. ADAPTATION OF ADULT MALE RATS TO NEAR-LETHAL DOSES OF HYPERTONIC SALT SOLUTIONS INJECTED INTRAPERITONEALLY

GROUP	SOL. INJ. PRIOR TO TEST DOSE	SOL. INJ. AS TEST DOSE	NO. OF RATS TESTED	NO. OF DEATHS FROM TEST DOSE	NO. WHICH HAD CONVULSIONS BEFORE DEATH	HOURS TO DEATH	% CHANGE IN BODY WT. DURING ADAPTATION OR DEADAPTATION PERIOD
1	0.5 M NaCl	0.5 M NaCl	15	2	2	2- 8	-2.5
2	0.154 M NaCl	0.5 M NaCl	3	3	3	48- 96	+10.2
3	Sham injection	0.5 M NaCl	14	10	7	3- 60	+4.3
<i>Retest of Survivors after a Period of Deadaptation</i>							
1		0.5 M NaCl	13	11	6	1-1080	+0.3
3		0.5 M NaCl	2	2		2- 32	-6.9

Data in table 6 and figure 2 show that at least part of the ability of rats to survive large doses of intraperitoneal concentrated salt solutions depends upon excretion of the salt at a certain high rate. The  $\text{Cl}^-$  concentrations of the urines of control and adapted groups do not differ. Greater  $\text{Cl}^-$  excretion in the latter depends on greater urine outputs as collected after spontaneous micturition in funnel cages. The table also shows that experimental survivors, after a period of 'deadaptation,' succumb to the previous dose, and their rates of water and  $\text{Cl}^-$  excretion are as low as those in the first test experiments of the unadapted controls.

*Cross Adaptation to Sea Water.* Ten male rats which had been on various voluntary intakes of sea water for several months were tested with lethal (16%) doses of sea water intraperitoneally. Three controls were similarly injected. All 13 animals died in from 1 to 9 hours without convulsions. No cross-adaptation to sea water given by different routes was demonstrated.

#### EFFECTIVE WATER INTAKES

*Drinking After Sea Water Administration.* Four rats were allowed fresh water *ad libitum* immediately after intraperitoneal injection of a 16 per cent dose of sea

water. None of the rats drank enough fresh water to save itself from death. Rats drank considerable fresh water after receiving 8 per cent of the body weight of sea water by stomach tube, but the drinking was spread over 6 hours. When the animals received a lethal dose of sea water their urges to drink were disturbed before effective drinking occurred. Rats given daily intraperitoneal injections of 12 per cent doses of hypertonic salt solutions drank more of a dilute milk mixture per 24 hours than did controls.

*Voluntary Sea Water Intakes in Rats.* To induce rats to drink sea water, whole dried milk was mixed with natural sea water in various proportions and supplied to the animals as the sole source of calories, with or without available fresh water. Table 7 gives data from 8 rats over 4-day periods on each of 3 different mixtures. The highest salt intake occurred with the lowest concentration of whole dried milk and with fresh water available. Weight was gained only on intake of the highest concentration of whole dried milk in sea water with fresh water available. The amounts of sea water taken by the rats under these forced conditions produced no

TABLE 6. WATER AND CHLORIDE EXCRETION RATES IN ADULT MALE RATS ADAPTED AND UNADAPTED TO HYPERTONIC SALT SOLUTION

GROUP	URINE COLLECTING TIME	NO. OF RATS	NO. OF DEATHS	MEAN URINE Cl <sup>-</sup> CONCEN.	MEAN URINE OUTPUT	MEAN Cl <sup>-</sup> EXCR.
	hr.			mEq/l.	ml/100 gm/hr.	mEq/100 gm/hr.
A	7.5	2	0	348	0.97	0.33
Control	7.5	7	5	367	0.47	0.17
Retest A	7.5	2	2	360	0.44	0.17
B	6.2	2	0	391	1.14	0.45
Control	6.2	3	3	384	0.69	0.27
Retest B	6.2	2	2	346	0.67	0.23

deleterious effects other than partial inanition, though the salt intake for each individual continued for 24 days.

Voluntary drinking of sea water was not appreciable (2% of body weight in 6 hours) in rats supplied with dry food, fresh water, and sea water. If then deprived of fresh water, scarcely more sea water was drunk. When animals were given repeated doses of distilled water by stomach tube, and others were given it by repeated intraperitoneal injections, both groups nearly to the point of water intoxication, there was no specific urge to drink sea water, though presumably it would have been to the animals' advantages to increase their salt intakes.

*Therapy Following Sea Water Injections.* Table 1 (group VI) shows the results of attempted therapy in rats following single intraperitoneal injections (16% of body weight) of natural sea water. Among 11 rats given single intraperitoneal injections of distilled water (10% of body weight) 1 to 1½ hours after sea water, there were only 3 deaths. Evidently if the peritoneal fluid is diluted soon enough, a majority of the animals can be saved from the lethal effects of the salt. In general, the concentration of salt given to rats by the intraperitoneal route is as important in the toxicity of the salt as the total amount given. Sucrose solution is more toxic than an equiosmolar salt solution or sea water when given intraperitoneally. The Mg<sup>++</sup> ion may be a modifying though not a prime factor in the toxicity of sea water given parenterally.

## EXPERIMENTS WITH OTHER SPECIES

**Mice and Guinea Pigs.** The lethal dose of sea water by intraperitoneal injection was essentially the same for adult mice and guinea pigs as for rats (table 1, group V). None of the guinea pigs had convulsions, but some of the mice showed violent 'shivering' movements before death. Four guinea pigs all died following single intraperitoneal injections of 1.0 M sucrose solutions in doses of 6 and 7 per cent of the body weight (table 1, group III). All of the animals had jerking movements, and their deaths occurred several hours later than in rats given sucrose solution in doses 14 to 18 per cent of the body weight. Hence no species differences were revealed.

**Dogs.** To test the effects of orally administered sea water on a larger mammal, possibly more similar to man in his physiology than rodents are, experiments were done on 2 female dogs weighing 8 and 9 kg. The animals received their last feeding 18 hours before each experiment was begun, and were allowed water *ad libitum* until restrained in stalls for the experiment. Woods Hole sea water at body temperature was given by stomach tube. Vomiting was common following the forced administra-

TABLE 7. VOLUNTARY INTAKES OF SEA WATER + MILK MIXTURES, WITH AND WITHOUT FRESH WATER *ad libitum*, EIGHT ADULT MALE RATS IN EACH GROUP

WHOLE DRIED MILK IN SEA WATER	MIXTURE DRUNK		SALT INTAKE		URINE VOL.		WT. AT END OF 4-DAY PERIOD	
	With None		With None		With None		With None	
	ml/100 gm.		gm/100 gm.		ml/100 gm.		% basal wt.	
%								
6.7	33	19	.98	.55	36	11	101	91
10.0	26	16	.76	.47	34	10	96	91
15.0	28	15	.75	.42	30	10	106	96

With = With fresh water. None = no fresh water.

tion of 5 per cent of the body weight of sea water at once; emesis occurred within 20 minutes. Vomiting could be delayed by giving the same amount in one per cent doses at half-hour intervals. The volume of vomitus was usually nearly as large as the immediately preceding dose of sea water, but its  $\text{Cl}^-$  concentration was approximately half that of sea water. Diarrhea was not a prominent feature after sea water was given orally. It never occurred during the period of observation, but the dogs frequently had watery stools after release from the stalls. Plasma  $\text{Cl}^-$  concentration in the dogs did not rise more than 50 per cent after forced ingestion of the moderate amounts of sea water given. No experiments on dogs were continued to the point of death. At the termination of the experiments, the dogs manifested intense thirst and drank large draughts of fresh water. During the following 24 hours it was not unusual for them to drink three times their control water intakes. Their appetites for food seemed unaffected by sea water.

**Seals.**<sup>8</sup> The studies on seals were motivated by the belief that if any mammals

<sup>8</sup> The investigation on harbor seals (*Phoca vitulina*) was carried out at the Mt. Desert Island Biological Laboratory during the summer of 1947. The seals were generously furnished by the Boothbay Harbor Station of the U. S. Fish and Wildlife Service.

should be found to have some natural resistance to sea water's toxic effects, they would most probably be marine mammals, especially the true seals, which live in sea water and, as far as is known, never have contact with fresh water (excepting certain land-locked species which inhabit fresh-water lakes in Canada and Asia). The experiments were carried out intensively on 2 male seal pups (yearlings) kept in a pen half-submerged in sea water. The animals weighed 30 and 31 kg., and maintained these weights on a daily ration of fresh iced herring. During experiments the animals were strapped to specially constructed boards indoors. Care was taken to keep the animals cool by wetting them. Sea water at body temperature was administered by stomach tube. Urine was collected by catheter, and blood samples were drawn from foreflipper veins.

One of the questions we sought to answer was, do seals drink sea water? Smith (24) found no evidence from seal urine or rectal washings that these animals swallow any considerable amount of sea water. Hiatt and Hiatt (25) showed that the seal's kidneys function more rapidly at times when food has yielded water to spare for excretion, and then decrease urine output sharply between meals, slowing the body's dehydration. Apparently seals can meet their water requirements without drinking fresh water. From our own observations we concluded that seals unavoidably swallow a little sea water when feeding, but this puts no stress on their homeostatic mechanisms. The animals were never seen to drink while in the pen in sea water.

One seal (*B*, fig. 3) was given 3.3 per cent of the body weight of distilled water in a single dose by stomach tube, after which  $\text{Cl}^-$  practically disappeared from the urine; yet when offered sea water hours later the animal drank only a swallow or two. If the seal was depleted of  $\text{Cl}^-$  it had no urge to replenish it by drinking sea water. Sea water in large doses (3.3% of body weight) apparently was just as irritating to the stomachs of some seals as it is to dogs, though individuals may vary in their tolerance of it. One seal vomited copiously 17 minutes after receiving a liter of sea water orally. Watery, green diarrhea began 9 minutes before the vomiting. The fluid lost by this animal in vomitus and feces was slightly more than the volume of sea water given; hence the net effect was dehydration. The  $\text{Cl}^-$  concentration of the vomitus was much below that of sea water (as in dogs). The animal easily retained a liter of distilled water; and it retained a liter of sea water given in 10 divided doses at half-hour intervals, though the latter caused some diarrhea. Another seal (*A*, fig. 3) retained a full liter of sea water by stomach, though diarrhea occurred. This animal drank fresh water when offered it after 8 hours.

Figure 3 shows that diuresis in seals is much greater after sea water administration than after distilled water. Attempts to instill sea water by rectal tube in a seal were futile, as the fluid was expelled as fast as introduced, and the resulting change in plasma  $\text{Cl}^-$  concentration was hardly measurable. Seals do not seem usually to concentrate  $\text{Cl}^-$  in their urine above that in sea water. In only 3 urine samples from our 2 seals were such high  $\text{Cl}^-$  concentrations found; these were very little above the  $\text{Cl}^-$  concentration of sea water and were not maintained. Sea water was infused into the seals' veins until death (table 8). Urinary  $\text{Cl}^-$  concentrations tended to decrease during the infusions. Death occurred very quietly, as from deep anesthesia. The heart gradually slowed but continued beating for some minutes after breathing

had ceased. The picture was like that described for  $Mg^{++}$  poisoning. Hence we succeeded in demonstrating no greater tolerance for sea water in the seal than in the rat.

#### DISCUSSION

*Clinical Observations.* Convulsions commonly followed intraperitoneal injections of hypertonic salt or sucrose solutions in rats, but rarely followed oral administration of salt solutions. In general, sea water convulsions did not suggest the progression

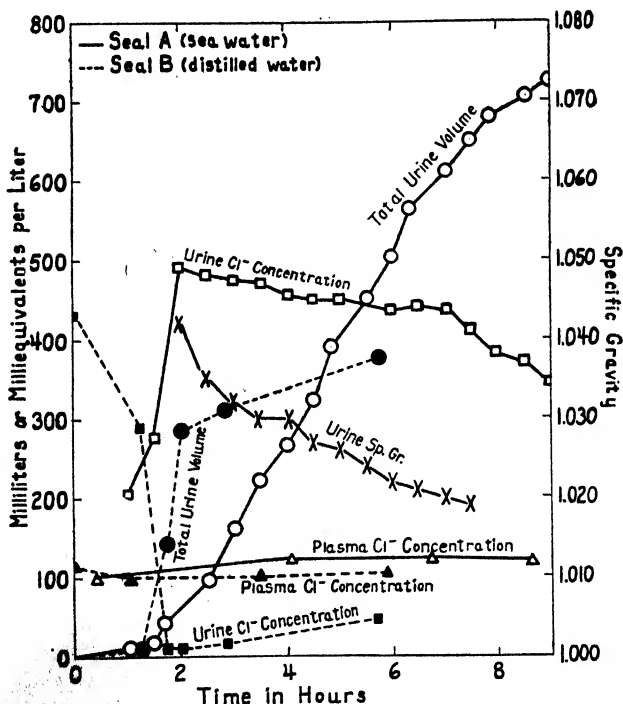


Fig. 3. OUTPUTS OF URINE AND CHLORIDE CONCENTRATIONS of urine and plasma, in two seals after forced ingestion of A) sea water or B) distilled water in doses of 3.3 per cent of body weight at zero time.

of epileptiform seizures. They consisted of an explosive hyperirritability of the rat, which ran about wildly if not closely confined. 'Convulsions' from sucrose or urea, in further contrast, resembled a shaking chill. Main (26) found, in determining the maximum lethal dose for NaCl injected subcutaneously, that NaCl alone produced an amazing hyperirritability in rats. On the other hand, Joseph and Meltzer (27) showed that infused NaCl killed dogs by stopping respiration, before circulation, without terminal convulsions, and asked whether this might not follow a curare-like action of the NaCl upon motor nerve endings. They got similar results with infused  $MgCl_2$ , though much sooner. Ulrich and Shternov (13) found that when

NaCl was given orally or intraperitoneally, as death neared respiration was permanently depressed before the heart was, and before this depression marked convulsions of a non-asphyxial type were common and appeared suddenly.

Respiratory distress was the commonest sign of disorder in our rats. Respiration was usually rapid and shallow after an intraperitoneal injection, then gradually became slower and gasping. The heart usually beat for 1 to 2 minutes after breathing had ceased.

Rats given lethal doses of salt solutions (or of water) by stomach tube became progressively weaker and flaccid before death. They had copious watery diarrhea and might evacuate more of a dilute fluid than the volume of salt solution given. Plasma  $\text{Cl}^-$  concentrations at death reached the lower limits seen at death from intraperitoneal sea water.

*Delayed Deaths from Sea Water or 0.5 M NaCl Solutions.* Many rats which survived for 24 hours after intraperitoneal injection of salt solutions and which were

TABLE 8. INTRAVENOUS INFUSION OF SEA WATER UNTIL DEATH IN TWO YOUNG SEALS

SEAL	BODY WT.	RATE AND DURATION OF INFUSION	PLASMA $\text{Cl}^-$ CONC., mEq/l.		HEMATOCRIT RATIO, % CELLS		URINE $\text{Cl}^-$ CONC., mEq/l.		SURVIVAL TIME FROM START OF INFUSION
			Pre-Infusion	Maximal <sup>1</sup>	Pre-Infusion	Minimal <sup>1</sup>	Pre-Infusion	Maximal <sup>1</sup>	
	kg.								minutes
A	29.3	1.1 l/1.7 hr., 1.5 l/1.1 hr.	106	176	64	45	239	439	171
B	27.3	0.1 l/0.1 hr., 1.0 l/0.5 hr.	120	203	58	44		537	77

Blood and urine were sampled every 30 minutes. <sup>1</sup> Values at death. <sup>2</sup> In seal A, 64 minutes after beginning infusion; in seal B, 3 minutes after.

then given fresh water and food failed to eat or drink again, and died after some days with marked inanition and obvious central nervous system involvement. Delayed death from sea water was never observed in rats which when given food and water at the end of 24 hours began to eat and drink again. Rats which survived oral doses of sea water always took fresh water when it was offered at the end of 6 hours or more after the sea water.

*Gross Pathology at Autopsy.* The commonest single finding at death from hypertonic salt solutions or sucrose was pulmonary congestion, with or without pulmonary edema. Following intraperitoneal injections of NaCl, sucrose or urea, there was evidence of widespread capillary damage, in the form of petechial hemorrhages, within the peritoneal cavity, especially in mesenteric and omental fat around stomach, spleen and pancreas; sometimes these petechiae were found in fat around the testes within the scrotum, into which peritoneal fluid leaked. We believe this capillary damage to be due to direct contact of the hypertonic solutions with the peritoneum. Occasionally areas of erosion were found within the stomach and intestine. The cause of this is not clear. Following stomach tube administration of hypertonic salt solutions, damage to gastric and intestinal mucosa was often severe.

*What Favors Survival of Some Individuals?* Rats that survived those intraperi-



toneal injections of hypertonic salt solutions which were lethal for at least 50 per cent of rats seemed often to be the individuals that excreted the most urine within the first 2 hours after injection (fig. 2). This was true whether the animals had been 'adapted' or not by previous sublethal injections; random control rats that survived were those which inherently performed as well as the adapted individuals. With more urine the survivors excreted more  $\text{Cl}^-$  (table 6). We are unprepared to name other factors which determine survival or death in individual animals given toxic doses of salt solutions by stomach. Survivors may have more or less diarrhea than non-survivors.

Men, dogs and seals will vomit sea water if they take enough of it into their stomachs (the rat does not vomit). Rats, dogs, seals and men have diarrhea from ingested sea water if there is no previous severe water deficit; in shipwrecked human beings who were quite dehydrated before resorting to drinking sea water, diarrhea has not been reported. Rats may have violent convulsions before dying from intraperitoneally injected sea water; these perhaps are similar to the violent actions of shipwrecked men who occasionally drink sea water, become wildly delirious, and hurl themselves overboard. On the other hand, cases have been cited (1) of human beings who became comatose and died quietly after drinking sea water. One such individual was little dehydrated before he drank sea water. Neither men nor seals can retain sea water for any length of time when it is administered by rectum, and sea water so instilled draws body fluid into it before it is expelled.

#### SUMMARY AND CONCLUSIONS

The lethal dose of sea water or of isosmotic  $\text{NaCl}$  solution for 50 per cent of rats, mice and guinea pigs when given the fluid by single intraperitoneal injection is  $16 \pm 2$  (S.D.) per cent of the body weight. The LD 50 of molar sucrose solutions for these species under the same conditions is less than half the LD 50 of equiosmolar salt solutions. The LD 50 of sea water by stomach tube in rats, given in divided doses at half-hour intervals, is essentially the same as by intraperitoneal injection. More gradual administration increases the total LD 50. The lethal chloride concentration of plasma is uniform for all administrations.

Dogs and seals retain more sea water by stomach if it is given in repeated small doses (0.33% of body weight) than if given in a single large dose (3.3% of body weight). Vomiting of the sea water is due to gastric irritation, not merely to distention by fluid. The total dose of i. v. sea water required to kill seals varies with the infusion rate, but is of the order of 4 to 9 per cent of body weight. Seals appear to be no more resistant to the effects of sea water than are other mammals. They do not excrete its salts any more concentratedly in urine.

Sea water may kill animals through cellular dehydration, while extracellular fluid volume is increased. But urea solutions probably kill without producing cellular dehydration. The central nervous system is believed most susceptible to tissue damage by sea water. The lethal dose of sea water for mammals is decreased by previous shortage of body water. Rats can be temporarily rendered more resistant to ordinarily lethal doses of sea water by administering repeated sub-lethal doses (adaptation).

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# ROLE OF UREASE IN THE GASTRIC MUCOSA<sup>1</sup>

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PREVIOUSLY, it was reported (1) that urease is primarily confined to those histological regions of the human gastric mucosa that contain parietal cells, and that the enzyme activity observed was proportional to the acid secretory response elicited by histamine; in the cancer stomach, in which acid secretion could not be provoked, no urease action was demonstrable. The possibility that urea hydrolysis is involved in acid secretion was considered, and for purposes of speculation a theoretical scheme was presented which related urea breakdown to acid production. To determine whether any such relationship actually exists the present investigation was instituted, particular attention being given to possible stoichiometric relations between the products of urea hydrolysis and acid formation.

Meanwhile the earlier report (1) stimulated Davenport and Jensen (2) to test the influence of added urea on the acid secretion by mouse stomach *in vitro*. No influence was observed but these investigators failed to consider the possible effect of the urea already present in the mucosa. During the preparation of this manuscript the proof of a note by Davies and Kornberg (3) was kindly sent by Dr. Davies in which this same point was brought out, and the latter authors studied the urea content of the gastric mucosae of frogs and also considered acid production in relation to urease activity. Points of agreement and disparity between their results and those herein presented are discussed.

Agreement with the previous observation that a physiological association exists between the degree of acid response and urease activity, regardless of whether the two are chemically related or just coincidentally operative, (1) appears in the work of Fitzgerald and Murphy (4). These workers observed that a meat diet, which could be expected to effect increased acid secretion, resulted in an increased urease activity in the cat stomach when compared to controls on a milk diet. It should be pointed out that the staining method of Sen used by Fitzgerald and Murphy in their attempt to localize urease in the gastric mucosa has been found unreliable by both Gomori, and Schwartz and Glick (5). Positive staining in tissues known to be devoid of the enzyme as well as a lack of reproducibility characterized the method.

The present experiments were conducted on the frog stomach actively secreting

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*in vitro*. The three approaches employed were: 1) Comparison of the rate and amount of acid secreted in media with and without added urea; 2) comparison of the rate and amount of acid secreted by mucosae with and without treatment by urease to remove endogenous urea; and 3) relation between rates of ammonia and HCl formation in the presence and absence of added urea.

#### EXPERIMENTAL

The *in vitro* stomach experiments were set up in two ways. In one, the whole mucosae from *Rana pipiens*, which were killed by pithing, were stripped off to give tubes that could be tied at each end to form secreting bags with the mucosal surface on the inside in the manner described by Davies (6). Following Davies' procedure throughout, a tied bag was placed in a Warburg vessel containing 5 ml. of Krebs-Henseleit bicarbonate-saline solution (7) which had been diluted 1.29 times with water and which contained 0.2 per cent glucose in the diluted liquid. The vessel was attached to a manometer for support and gassed with 95 per cent oxygen-5 per cent carbon dioxide for 7 minutes at room temperature. Then the vessel was immersed in a 26° constant temperature bath for 5 to 7 hours, after which the mucosa bag was removed, opened and the secretion that had collected within was transferred to a tarred weighing bottle. The secretion weight and the dry weight of the mucosa were determined, the latter by drying at 110° to constant weight.

In the other *in vitro* secreting system, sheets of gastric mucosa from bullfrogs (*Rana catesbiana*), which were killed by pithing, were stripped from the musculature after opening the stomach along the lesser curvature, rinsed with physiological saline, and mounted in the apparatus shown in figure 1. This device is similar to those employed by Crane *et al.* (8) and Patterson *et al.* (9), and it enabled pH measurement on each side of the mucosa wall and removal of aliquots for analysis. The apparatus was not thermostated, but the temperature was under 30°, above which heat damage to the tissue occurs according to Davies (6). The temperature of each experiment was recorded.

In those experiments designed to test the effect of the urea present in the tissue, 10 ml. of 0.65 per cent sodium chloride was placed in the compartment on the mucosal side, figure 1, and to the serosal compartment was added 10 ml. of a Krebs' phosphate-saline solution as modified by Patterson and Stetten (9) and then diluted to isotonicity for the frog (71 ml. diluted with water to 100 ml.). Oxygen passed through soda lime and then water was bubbled through the solutions as indicated in figure 1. Tissue urea was removed by placing the tissue in a urease solution for 45 minutes at room temperature. The urease solutions used were either a 5 per cent solution of purified jack bean urease (Arlington Chemical Company, Yonkers, New York) in 15 ml. of the medium used in the serosal compartment of the apparatus, or a solution of 2 ml. of crystalline jack bean urease in 50 per cent glycerol (Worthington Biochemical Laboratory, Freehold, New Jersey) plus 15 ml. of the medium. The latter solution was subjected to 60° for 5 minutes to activate the enzyme according to Hofstee (10). After the urease treatment the mucosae were washed in 0.65 per cent sodium chloride and mounted in the apparatus. The urea content of the tissue was determined on the material trimmed from the piece used in the apparatus; urea analysis was performed

immediately or the tissue was kept frozen with dry-ice until time for analysis. After a secretion experiment the dry weight of the tissue was determined by cutting out the functioning tissue disc and drying to constant weight in a vacuum oven at 55°.

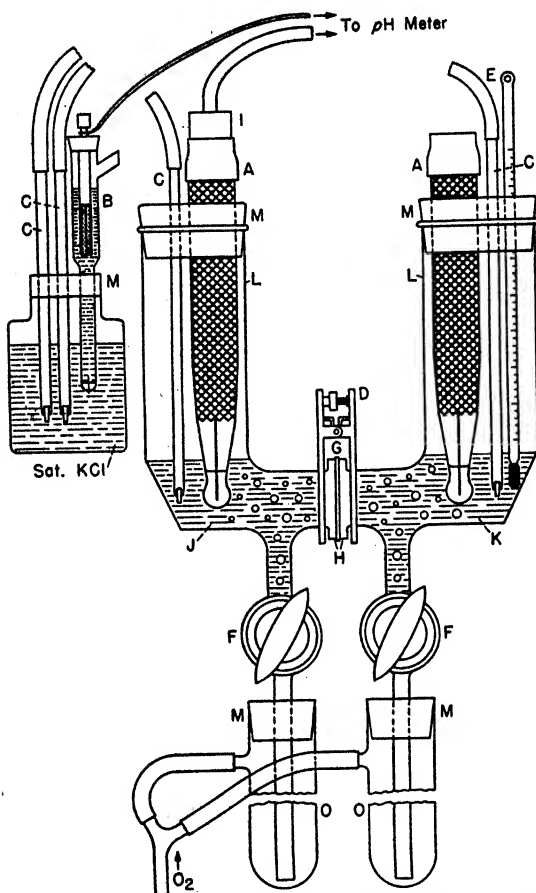


Fig. 1. APPARATUS FOR CONTINUOUS STUDY of secreting gastric mucosa. A) Shielded glass electrodes, #13459, Cambridge Instrument Co., Ossining, N. Y.; B) calomel electrode, #14774, Cambridge Instrument Co.; C) saturated KCl bridge with ground glass stopper at tip; D) clamp, #3241, size 28, Arthur H. Thomas Co., Philadelphia; E) thermometer; F) stopcocks, 2-mm. bore; G) mounted mucosa; H) ground glass surface to grip mucosa; I) detachable lead to electrode; J) and K) media, see text; L) cell proper (70 per cent of actual size) M) rubber stoppers; O) traps.

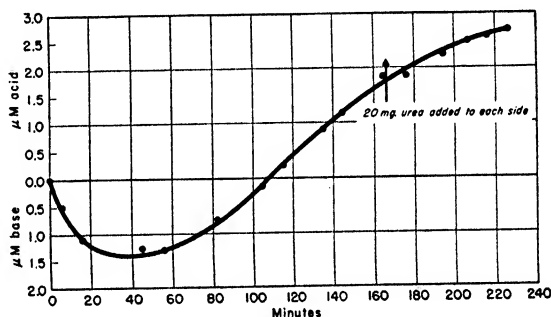
In those experiments in which the rate of acid production was followed in the presence and absence of added urea, the apparatus, figure 1, was used with 10 ml. of the modified Krebs' phosphate-saline solution in each of the two compartments.

**Measurement of Acid.** In the experiments with the bags of mucosa, the weighed secretion from each bag was diluted with 2 to 3 ml. of distilled water and the free and

total acid was determined by titration with 0.100N NaOH to pH 4.0 and 7.4 using a pH meter with glass electrode. A micro burette was employed with graduations down to 0.1  $\mu$ l. In the experiments with the mucosa sheets in which the acid formed was followed continuously, the acid was determined from pH measurements which were referred to standard titration curves relating pH and mEq. of HCl or NaOH added to the media used in the compartments of the apparatus, figure 1. As control experiments, aliquots of solution from the mucosa compartment were back titrated to the original pH with the result that the amount of acid calculated from this titration corresponded to the quantity determined by pH measurement. Hence effects of secretion of buffer substances were negligible in these experiments. In most of the experiments with mucosa sheets, however, the acid formed in the mucosa compartment was determined by titration of 2 ml. aliquots to pH 7.0 with 0.0274N NaOH.

*Measurement of Ammonia.* In the experiments with mucosa bags, ammonia was determined in the secretion after it had been titrated for acid content. The liquid was transferred into the outer well of a Conway cup (11) having an outer diameter of 7

FIG. 2. SECRETION BY ISOLATED FROG gastric mucosa with and without added urea.



cm., 2 ml. of 0.006N  $\text{H}_2\text{SO}_4$  was placed in the center well, the ammonia was liberated by adding one ml. of 2.5N NaOH to the outer well, the cup was sealed, and the diffusion into the acid was allowed to proceed for 3 hours at room temperature. The ammonia absorbed in the inner well was then determined by the colorimetric method of Russell (12). In the same fashion, ammonia measurements were also carried out on 2- or 3-ml. aliquots of the medium surrounding the mucosa bags.

*Measurement of Tissue Urea.* The colorimetric xanthidrol method of Engel and Engel (13) was used for urea determination. It was found that commercial preparations of xanthidrol were often unsatisfactory for this purpose, even after several recrystallizations. The problem was solved by preparing the compound in our own laboratory by reduction of xanthone with sodium amalgam, (14). Xanthidrol solutions were not used after one week. To avoid possible loss of urea by the action of urease in the tissue, the sample was lyophilized, and brought to constant dry weight by subsequent heating at 55° in a vacuum oven. The dried sample was either ground in a Wiley mill or cooled with dry-ice and pulverized in a Plattner's diamond mortar. A protein-free filtrate was prepared from a weighed sample by extraction overnight with a solution of 5 ml. water, one ml. 0.66N  $\text{H}_2\text{SO}_4$ , and one ml. 10 per cent sodium

tungstate, followed by clarification with  $\text{CuSO}_4$  and  $\text{Ba(OH)}_2$  (15). The filtrate was then analyzed for urea by the Engel procedure.

## RESULTS

It is apparent from figures 2 and 3 and table 1 that addition of urea to the medium surrounding the sheets or tied pouches of mucosa, either at the start or during a secretion experiment, had no effect on the initial rate of acid production but did reduce the average rate over the entire 5- to 7-hour experimental period. Part of the decrease in the acid resulted from increased neutralization by ammonia which was liberated on urea hydrolysis. This ammonia was determined, table 1, and if a correction

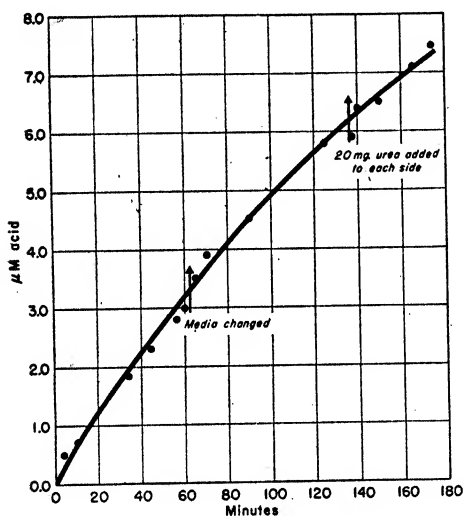


Fig. 3. EFFECT OF CHANGING MEDIA and adding urea on secretion by isolated frog gastric mucosa

is applied to account for the neutralization effect, the average free and total acid values of  $102 \pm 36$  and  $125 \pm 31$  become  $112 \pm 30$  and  $135 \pm 32$ , respectively; while in the presence of added urea the corresponding values of  $51 \pm 13$  and  $73 \pm 14$  become  $68 \pm 14$  and  $90 \pm 15$ , respectively. Thus, even the corrected rate of secretion of both free and total acid was reduced as the result of the presence of 100 mg. per cent urea in the surrounding medium.

It will be noted in figure 2 that liberation of alkali preceded the acid secretion, while this was not the case in the experiment shown in figure 3. The course shown in figure 2 was obtained in 35 out of 41 cases.

From table 1 it is also clear that the molar ratio of either free or total acid to the ammonia formed in either the secretion, the surrounding medium, or both is always greater than one. Of course the ratio is reduced in the presence of added urea since liberation of ammonia is then increased. The ammonia formed in the secretion averaged four times that produced in the surrounding medium, and in the presence of 100 mg. per cent urea in the latter there is still about 2.7 times as much ammonia in the secretion.

The data in table 2 were obtained from the experiments on mucosa sheets mounted in the apparatus, figure 1. The molar ratio of acid secreted to tissue urea in experiments with untreated mucosae was always greater than one. A tendency to lower ratios during the initial course of secretion, and much higher values later, results from an early loss of the urea store. In a control experiment on part of the tissue used in experiment 46 it was found that over half of the tissue urea had diffused into the medium in 90 minutes. After tissue urea was removed by prior treatment with urease,

secretion of acid continued. Control experiments are included in which the pre-treatment was repeated without urease; in these it may be seen that the lower rates of acid production were found in the presence of added urea in the medium. It is clear from data in this table that no stoichiometric relation exists between acid secreted and tissue urea. Histamine was found to have no consistent effect on acid secretion in these experiments (see ref. 6).

## DISCUSSION

Davies and Kornberg (3) could detect no ammonia in 3-hour secretions of isolated gastric mucosae of frog and concluded that any acid-neutralizing role for the

TABLE 1. RELATIONSHIP BETWEEN ACID AND AMMONIA SECRETED BY TIED POUCHES OF FROG MUCOSA IN THE PRESENCE AND ABSENCE OF UREA

GM. SECRETION /GM. DRY WT. TISSUE	μM FREE HCl/GM. DRY WT. TISSUE/HR.	μM TOTAL HCl/GM. DRY WT. TISSUE/HR.	μM SECRETION NH <sub>3</sub> /GM. DRY WT. TISSUE/HR.	FREE HCl/ SECRETION NH <sub>3</sub> <sup>1</sup>	TOTAL HCl/ SECRETION NH <sub>3</sub> <sup>1</sup>	SECRETION NH <sub>3</sub> / MEDIUM NH <sub>3</sub> <sup>1</sup>	FREE HCl/ MEDIUM NH <sub>3</sub> <sup>1</sup>	TOTAL HCl/ MEDIUM NH <sub>3</sub> <sup>1</sup>	FREE HCl/ TOTAL NH <sub>3</sub> <sup>1</sup>	TOTAL HCl/ TOTAL NH <sub>3</sub> <sup>1</sup>
<i>No Urea in Surrounding Medium</i>										
5.0	19	31	4.7	4.0	6.8	3.7	15	25	3.2	5.4
14.8	186	216	18.3	10.2	11.8	6.2	63	73	8.7	10.0
12.6	73	100	14.2	5.1	7.7	3.1	16	24	3.9	5.8
7.0	65	78	5.5	11.8	14.2	2.6	31	37	8.6	10.3
12.0	160	188	8.6	10.6	21.9	4.6	90	100	16.2	18.1
10.3 ± 2 (25) <sup>2</sup>	102 ± 36 (25)	125 ± 31 (21)	10.2 ± 2.5 (9)	10.1 ± 2.7	12.5 ± 2.5	4.0 ± 0.7	43 ± 13	52 ± 15	8.1 ± 2.1	9.9 ± 2
9.0 ± 0.6	91 ± 11	114 ± 11	8.2 ± 1.6							
<i>100 mg. % Urea in Surrounding Medium</i>										
6.1	17	33	11.5	1.4	2.9	3.5	5	10	2.0	4.0
10.0	103	122	22.6	4.5	5.4	3.6	16	20	3.6	4.2
12.3	48	87	13.8	3.4	6.2	0.3	1	19	0.8	1.5
4.5	42	54	23.6	1.8	2.3	2.3	4	5	1.2	1.6
8.5	45	70	14.7	3.0	4.7	4.0	12	19	2.4	3.8
8.3 ± 1 (27) <sup>2</sup>	51 ± 13 (27)	73 ± 14 (23)	17.2 ± 2.3 (9)	2.8 ± 0.5	4.3 ± 0.7	2.7 ± 0.6	8 ± 3	15 ± 3	2.0 ± 0.4	3.0 ± 0.6
9.1 ± 0.6	75 ± 9	104 ± 13	22.2 ± 4.1							

<sup>1</sup> Molar ratios. <sup>2</sup> Numbers in parentheses denote no. cases.

tissue urease was excluded in their experiments. The data in table 1 indicate that the ammonia derived from urea can be appreciable and it can influence the acidity of the secretion. Sumida (16) found that perfusion of dog stomach with Locke's solution containing added urea resulted in a secretion containing increased ammonia compared to controls without the urea, and Fitzgerald and Murphy (4) reported that the ammonia in human gastric juice was proportional to the blood urea concentration. The latter investigators stressed the possibility that the enzymatic hydrolysis of urea is of importance in regulating the acidity of the gastric juice and in protecting the mucosa from the action of acid-pepsin. This acid-neutralizing role of ammonia derived from urease activity has also been considered by others (17, 18). The decrease in acid secreted *in vitro* as a result of the presence of added urea cannot be accounted for entirely by the ammonia in the secretion. This is in accord with the *in vivo* findings of



Fitzgerald and Murphy (4) on humans. Whether the effect is due to resorption of part of the ammonium chloride, as suggested by the latter authors, remains to be established.

It also follows from the present investigation that urease activity is not likely to be directly concerned in acid formation since no stoichiometric relationship could be demonstrated between the quantities of acid elaborated and urea hydrolyzed.

TABLE 2. RELATIONSHIP OF TISSUE UREA AND HYDROCHLORIC ACID SECRETED IN VITRO BY ISOLATED GASTRIC MUCOSAE OF BULLFROG (*Rana catesbiana*)

EXPER. NO.	TEMP.	TISSUE DRY WT.	TISSUE UREA	HCl SECRETION				$\mu\text{M}$ HCl/ $\mu\text{M}$ TISSUE UREA	REMARKS
				Total HCl	Interval	$\mu\text{M}/\text{cm.}^2$ tissue	$\mu\text{M}/\text{hr}/\text{gm. dry wt. tissue}$		
	$^{\circ}\text{C.}$	mg.	% dry wt.	$\mu\text{M}$	min.				
<i>Mucosae Mounted in Cell Immediately After Removal</i>									
28 <sup>1</sup>	24	48	0.16	1.8	5-63	1.2	40	1.4	
45 <sup>2</sup>	22	31	0.17	4.0	14-180	0.9	45	4.5	
46 <sup>2</sup>	25	37	0.11	3.8	0-90	1.7	68	5.6	
			0.048	3.0	90-180	1.3	54	10.	
			0.048	6.2	90-300	1.2	48	21.	
<i>Mucosae Allowed to Stand in Medium for 45 min. Before Mounting in Cell</i>									
44 <sup>2</sup>	21	26	0.35	1.7	50-230	0.4	22	1.1	50 mg. % urea in medium
48 <sup>2</sup>	25	32	0.23	1.1	20-60	1.1	51	0.9	medium without added urea
49 <sup>2</sup>	24	40	0.24	1.9	10-127	0.7	25	1.2	100 mg. % urea in medium
				2.8	10-240	0.5	18	1.8	
<i>Mucosae Allowed to Stand in Medium Containing Urease for 45 min. Before Mounting</i>									
17 <sup>2</sup>	23	42	0.018	3.2	40-206	0.8	27	24	5% Arlco urease in medium
43 <sup>2</sup>	23	95	0.004	3.7	60-310	0.6	9	55	2 ml. Worthington cryst. urease in glycerol added to 15 ml. medium
47 <sup>2</sup>	25	51	0.002	5.2	80-240	1.3	38	320	5% Arlco urease in medium
50 <sup>2</sup>	25	49		3.3	43-143	1.3	41		5% Arlco urease in medium
51 <sup>2</sup>	25	37	0.017	4.8	30-245	0.9	36	46	

<sup>1</sup> 0.1 mg. histamine subcutaneously 15 min. before removal of mucosa. <sup>2</sup> Histamine not used.

<sup>3</sup> 0.05 mg. histamine in solution in each cell compartment.

However it is striking that in humans a parallelism seems to obtain between the acid secretory response to histamine and the gastric urease activity (1). From the evidence at hand, it would appear that a physiological relationship exists between the acid secreting mechanism and the urease activity, and although the urease is not needed for acid production, the two may be associated to enable the urease to act in a regulatory manner on the gastric acidity, and also to function in a protective capacity within the mucosa to prevent acid-pepsin damage. The urease function occurs primarily within the mucosa where the conditions of pH etc. are more favorable; and

its role in the secreted juice is normally a minor one since, not only is the pH too low, but peptic digestion can destroy the urease as Hollán (19) has emphasized.

#### SUMMARY

Studies on the influence of urea on frog gastric mucosae secreting *in vitro* revealed that no stoichiometric relationship could be demonstrated between urea hydrolysis and acid secretion. Therefore it is unlikely that urease activity is directly involved in the acid production. Evidence has been presented which is interpreted as indicating that urease, by releasing ammonia, has a role in regulating gastric acidity and in protecting the mucosa against the action of acid-pepsin.

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# PYRIDOXINE AND EXPERIMENTAL X-RADIATION INJURY IN RATS

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IT HAS been reported that pyridoxine is beneficial therapy in the relief of radiation sickness in humans (1-5). Goldfeder *et al.* (6) found that either folic acid or pyridoxine given intramuscularly to mice for 7 days before x-radiation and for 13 days afterward, extended significantly the life-span of the irradiated mice. However, no effect of these vitamins on the leucopoietic system was observed. If the action of pyridoxine is related to its role as a vitamin, it may be suggested that patients or animals responding to pyridoxine therapy were in a state of sub-optimal pyridoxine nutrition, either dietary in origin or due to destruction of pyridoxine *in vivo* by x-radiation. If loss of tissue pyridoxine were accelerated in irradiated animals, it might be anticipated that the severity of the effects of dietary pyridoxine-deprivation would be augmented by exposure to x-rays and that biochemical signs of pyridoxine insufficiency might be detected in irradiated animals not deprived of pyridoxine. Studies in our laboratory have indicated that pyridoxine deprivation has an effect upon the blood urea of rats (7) and that a similar effect on blood urea was evident in human cases of hyperemesis gravidarum (8). These observations suggested that the effectiveness of pyridoxine in radiation injury in rats could be studied by measurements of blood urea. It was also considered useful to determine the amount of vitamin B<sub>6</sub> in the livers of irradiated and of non-irradiated rats to ascertain whether radiation caused some destruction of the vitamin B<sub>6</sub> with a consequent endogenous insufficiency. This investigation was conducted to obtain basic information prior to a study of radiation sickness in humans.

## METHODS

All animals used in this study were young albino rats. They were housed individually in screen-bottom cages with water freely available. The experimental groups consisted of 8 to 12 animals with the sexes equally represented. The compositions of the pyridoxine-deficient diets are described in table 1. Control animals received a daily supplement of 40  $\mu$ g. of pyridoxine hydrochloride; this was contained in 0.5 ml. of solution and was added to the food.

After a preparatory period during which the deprived animals developed symptoms of pyridoxine deficiency, total body exposure to x-rays was carried out. At the end of a post-irradiation interval, the rats were fasted for 15 hours and then anesthetized with Nembutal (sodium pentobarbital) for withdrawal of blood from the heart

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and for removal of the liver. Leucocyte counts, as well as determinations of hemoglobin and urea concentrations, were carried out on the individual blood samples. Hemoglobin was determined by the cyanmethemoglobin method as described by Collier (9), and urea by the procedure of Archibald (10). The livers of the members of each group were pooled for measurement of the total vitamin B<sub>6</sub> content; the yeast growth assay of Atkin *et al.* was employed (11).

Irradiation of the animals was carried out in the Department of Radiology of the Toronto General Hospital. The rats were held in compartments of perforated plastic boxes and were exposed in groups of 5 or 6. All doses were administered in a single exposure. Use of the following physical factors; 200 kv., 20 ma., 50-cm. STD, 20 x 20-cm. field, 0.25 mm. Cu filter, resulted in the delivery of approximately 33 roentgens per minute. In preliminary investigations, the effects of exposure of normal animals receiving the stock diet (Purina Fox Chow) were studied. The results were in essen-

TABLE 1. COMPOSITION OF PYRIDOXINE-DEFICIENT DIETS

DIETARY CONSTITUENT	PERCENTAGE IN DIET				
	A	B	C	D	E
Sucrose (commercial).....	54		44	74	59
Casein (SMACO, vitamin-free).....	20	90	46	16	21
Corn oil (Mazola).....	20				10
Salts (Steenbock 40).....	4	4	4	4	4
Agar (British Drug Houses).....	2	2	2	2	2
Vitamin powder <sup>1</sup> .....		4	4	4	4

<sup>1</sup> The vitamin powder was prepared by incorporating the following into 800 gm. of vitamin-free casein: 100 mg. of thiamine chloride, 100 mg. of riboflavin, 400 mg. of calcium pantothenate, 400 mg. of nicotinic acid, 20 mg. of biotin, and 20 mg. of pteroyl-glutamic acid. All diets also contained 2 gm. of choline chloride, 2 gm. of inositol and 0.15 gm. of a cod liver oil concentrate (Ayerst, McKenna and Harrison no. 33101) per kg. of diet. In the series in which *diet A* was fed, the vitamins were injected subcutaneously each day, in amounts equivalent to those supplied by 5 gm. of the other rations.

tial agreement with observations reported by Prosser *et al.* (12) and Johnson *et al.* (13). Although dosages of 50 r and 150 r did not affect the rate of weight gain of the rats during an 18-day observation period, the weight increments of animals exposed to 250 r were subnormal. Most rats receiving 500 r developed diarrhea and loosening of the fur, and exhibited sharp weight declines in the first two days after irradiation. Subsequently, gains occurred but a second decline was evident between the 10th and 13th days after exposure. Administration of 750 r caused precipitous weight losses and was completely lethal within 8 days. In a second preliminary series, groups of animals receiving 450 r were sacrificed at intervals from 2 to 15 days after irradiation. Leucocyte counts fell from an initial average value of 6.8 to 0.8 thousand per cu. mm. on the second day and to still lower levels on the fourth day. This severe leucopenia persisted throughout the observation period. A decrease in hemoglobin was first apparent on the 6th day after exposure, and fell to a minimum of 3.8 gm/100 ml. of blood on the 13th day. On the basis of these preliminary studies, the x-ray dosage levels and post-irradiation intervals were selected for the experiments to be described.

## RESULTS

*Diet A* was employed in the first series in which the effects of x-radiation on rats receiving pyridoxine were compared with the responses of animals deprived of this vitamin. At the end of a preparatory period of 5 to 6 weeks, during which typical dermal manifestations of pyridoxine deficiency developed, comparable groups of rats were exposed to dosages of x-rays from 100 r to 500 r. Groups of control animals were similarly exposed after a 10-day period in which they were provided with the deficient diet supplemented with pyridoxine. All rats were sacrificed 7 days after irradiation. The observations are summarized in table 2.

Inspection of the data in table 2 showed that x-ray dosage above 300 r had a definite effect on body-weight gain and on the leucocyte count but had no effect on

TABLE 2. EFFECTS OF IRRADIATION ON PYRIDOXINE-DEPRIVED AND CONTROL RATS

DIETARY PYRIDOXINE	DOSAGE	AV. WT. CHANGE AFTER EXPOSURE	AV. BLOOD VALUES			AV. LIVER TOTAL B <sub>6</sub>
			Hemoglobin	Leucocytes	Urea	
	r	gm.	gm/100 ml.	th/cw. mm.	mg/100 ml.	µg/ gm. moist tissue
+	0		12.7	7.64	35.3	12.7
-	0		12.3	5.26	29.1	4.9
+	100	+26	12.5	3.30	31.1	10.5
-	100	0	13.5	2.04	26.4	4.4
+	200	+14	11.7	1.70	41.0	12.2
-	200	+3	13.0	1.14	32.8	4.9
+	300	+18	11.5	0.92	33.3	11.5
-	300	-3	12.9	0.69	31.2	4.3
+	350	+2	9.9	0.53	31.3	12.0
-	350	-1	11.6	0.34	28.3	4.3
+	400	+9	9.3	0.56	32.3	11.4
-	400	-2	12.7	0.81	31.2	4.9
+	500	+3	9.7	0.31	33.8	11.1
-	500	-14	11.5	0.26	28.6	4.8

blood urea or on the quantity of vitamin B<sub>6</sub> in the liver. The continued provision of pyridoxine made possible either a greater weight gain or a maintenance of body weight after radiation, caused an expected larger storage of vitamin B<sub>6</sub> in the liver but did not prevent the serious drop in leucocyte count. It should be noted that pyridoxine-supplemented rats receiving more than 300 r showed a subsequent decrease in hemoglobin, while pyridoxine-deprived animals did not.

It has been demonstrated in several laboratories that the feeding of high protein diets augments the severity of pyridoxine deficiency (14, 15) and it is now generally accepted that compounds of the vitamin B<sub>6</sub> group are intimately involved in certain phases of amino acid metabolism. In view of these considerations, an experiment similar in design to the preceding one was carried out but in which a diet very high in protein (*diet B*) was employed. Again however, the observations did not suggest that the damage associated with a given exposure to x-rays was intensified if the animals had been rendered pyridoxine-deficient. The severity of the leucopenia induced at each

exposure level was very similar in deficient and control rats. In harmony with previous experience in this laboratory (7), the blood urea concentrations of the deficient animals fed the high casein diet were elevated. However, the extent of this rise was no greater in radiated than in non-irradiated rats, and was similar in all deprived animals, bearing no relationship to the duration of the exposure to which they had been subjected.

In a further experiment, the responses of pyridoxine-deficient and control rats exposed to 500 r were compared using three different levels of dietary casein (*diets B, C, and D*). Four groups of animals were maintained on each diet: an unsupplemented deficient group fed *ad libitum*, a control group receiving pyridoxine but fed isocalorically with the deficient group, and two control groups fed *ad libitum*. Since

TABLE 3. EFFECTS OF EXPOSURE TO X-RAYS ON PYRIDOXINE-DEFICIENT AND CONTROL RATS FED DIETS WITH DIFFERENT CASEIN CONTENTS

CASEIN IN DIET	DIETARY PYRIDOXINE	EXPOSURE	AV. WT. CHANGE AFTER EXPOSURE	AV. BLOOD VALUES		AV. LIVER TOTAL B <sub>6</sub>
				Leucocytes	Urea	
%		r	gm.	th/cu. mm.	mg/100 ml.	μg/gm. moist tissue
94	—	500	—19	0.60	61.3	11.6
	+ (paired)	500	—11	0.25	62.8	14.5
	+	500	—23	0.25	40.6	12.8
	+	600	—22	0.48	48.4	14.9
50	—	500	+3	0.42	32.2	7.5
	+ (paired)	500	+1	0.35	39.4	15.0
	+	500	—18	0.55	29.0	13.0
	+	600	—28	0.10	33.1	13.5
20	—	500	—16	0.28	34.6	7.4
	+ (paired)	500	—13	0.18	24.6	12.4
	+	500	—12	0.40	26.7	11.3
	+	600	—2	0.33	26.2	12.1

the latter animals gained more weight than the first two groups, it was felt that their somewhat greater body size might affect the extent of penetration of the radiation into the tissues of the abdominal area. In an effort to circumvent this possible source of error, the exposure of one control group on each diet was increased to 600 r. The results of this experiment are summarized in table 3. As in the previous series, no indication was obtained that the deficient animals, whether receiving a diet of normal or excessive protein content, were more susceptible than control animals to x-ray injury.

In a final series, pyridoxine-deficient rats and animals receiving this factor were exposed to 500 r and their responses to this dosage compared at both 6 and 21 days after its administration. In addition, the influence of pyridoxine therapy during the post-irradiation period was examined. In this experiment all animals were provided with diet E *ad libitum*. The results are given in table 4. Examination of the data obtained from the groups killed 21 days after exposure suggests that the rate of recovery from the leucopenia was somewhat retarded in animals which were pyridoxine de-

ficient at the time of exposure. The post-irradiation body weight changes of the control animals appeared to have been favorably affected by the injection of extra pyridoxine. Apart from these observations however, no striking evidence of differences in the susceptibilities of deficient and supplemented rats to irradiation was apparent.

#### DISCUSSION

In interpreting the reported data, no attempt has been made to attach significance to small differences between mean values for various groups of animals. Standard deviations for all means were calculated. In the case of hemoglobin values the standard deviations were always less than one fifth of any mean. Standard deviations of mean

TABLE 4. RESPONSES OF PYRIDOXINE-DEPRIVED AND CONTROL RATS TO POST-IRRADIATION PYRIDOXINE THERAPY

PYRIDOXINE		AV. WT. CHANGE AFTER EXPOSURE	AV. BLOOD VALUES		
Dietary	After Exposure <sup>1</sup>		Leucocytes	Hemoglobin	Urea
		av. gm.	th/cu. mm.	gm/100 ml.	mg/100 ml.
<i>Post-Irradiation Period 6 Days</i>					
—	—	—18	0.15	12.7	36.6
—	+	—8	0.28	11.3	35.7
+	—	—14	0.21	12.1	20.6
+	+	—19	0.32	11.9	28.3
<i>Post-Irradiation Period 21 Days</i>					
—	—	—2	1.04	9.5	35.8
—	+	+37	1.41	8.7	31.1
+	—	+2	1.94	10.4	26.8
+	+	+15	2.18	9.4	32.0

<sup>1</sup> Pyridoxine therapy consisted of the daily subcutaneous administration of 200 µg. of pyridoxine hydrochloride in saline to each animal.

values for body weights, leucocyte counts and blood urea values were always less than one third of the respective means.

Under the experimental conditions which were employed in these investigations, the degree of injury sustained by rats receiving severe total body irradiation did not appear to bear a relationship to their state of pyridoxine nutrition. The effects of exposure were not accentuated by previous pyridoxine deprivation, and measurements of the hepatic stores of total vitamin B<sub>6</sub> did not indicate that loss of this factor had been increased in irradiated animals. It is not possible to compare our results with those of Goldfeder *et al.* (6) because different species of animals were used and the experimental procedures were not the same.

#### SUMMARY

To investigate a possible relationship between pyridoxine insufficiency and x-ray injury, the effects of total body irradiation on pyridoxine-deficient rats and on ani-

imals receiving this factor were compared. Pyridoxine deprivation was not found to increase the susceptibility of rats to x-ray damage and irradiation did not affect the concentration of total vitamin B<sub>6</sub> in the livers of either deficient or control animals.

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# CHEMICAL AGENTS INFLUENCING SURVIVAL TO 'EXPLOSIVE' ANOXIA

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MANY drugs have been studied in relation to anoxic resistance. Many of the reports have been those embodying different rates of decompression or step-like decompression to a lethal (simulated) high altitude, the animal's resistance to anoxia being measured quantitatively by either time required to die or highest simulated altitude reached by the animal. Recently, rapid or so-called 'explosive' decompression has become more widely used. The influence of the chemical substance on anoxic survival may vary with the technique used as will be apparent from a comparison of our findings with those of others. For example, a drug which requires a long time for its main pharmacological action might and should react differently if the animal is instantly decompressed instead of being slowly decompressed. During a period of prolonged slow decompression, time allows the animal to become somewhat acclimatized possibly by such means as polypneic breathing, lowering of body temperature, water loss, vasomotor changes, blood sugar changes, etc. (1, 2). Doubtless the main reason for early work employing slow decompression techniques was simulation of airplane ascent. There such factors as rate of decompression became very pertinent and the difficulty of comparing results from different laboratories and of duplicating procedures often became well nigh impossible. Explosive decompression occurring in a fraction of a second reduces these differences to a minimum.

## METHOD

An animal chamber consisting of a bell jar was attached to a larger jar serving as a ballast chamber. The ballast jar was evacuated by a vacuum pump to 25 mm. Hg pressure. The animal was then placed in the animal chamber and a large bore stopcock connecting the two chambers opened. In less than a second the pressure in the animal chamber was 'explosively' reduced to 105 mm. Hg (equivalent to about 47,000 feet, or to 3% oxygen at sea level). At the same instant a stopwatch was started and survival measured in seconds. It is interesting to note that the anoxic anoxia of 105 mm. Hg is as complete as that of 0 mm. Hg in the mouse (3). The drugs were administered intraperitoneally and the dose chosen for best pharmacological effects on the basis of earlier work or calculated from recorded LD<sub>50</sub> doses (4). In certain cases doses were chosen by experimentation. The interval of time between

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injection and decompression was gauged by experimentation and in some cases by subjective visible behavior signs such as piloerection, excitability, equilibrium changes and breathing.

### RESULTS AND DISCUSSION

The results are shown in tabular form. Two controls are shown since the mice used were of two strains each having different average survival times, *A* surviving

TABLE 1. EFFECTS OF 16 CHEMICAL AGENTS ON SURVIVAL TIME OF MICE SUBJECTED TO ANOXIC ANOXIA PRODUCED BY EXPLOSIVE DECOMPRESSION OF 105 MM. HG

CHEMICAL SUBSTANCE	DOSAGE	NO. OF MICE	INTER-VAL	SURVIVAL TIME		STAND-ARD ERROR $\pm$	PERCENT-AGE FROM NORMAL
				min.	sec.		
<i>Controls A</i>		66			37.4	1.07	
Ethanol 32%	10 ml/kg.	15	30		58.3	7.01	+55.8
	20 ml/kg.	15	30		103.6	7.55	+177.0
Methanol 32%	10 ml/kg.	10	15		37.8	2.34	+1.0
	20 ml/kg.	10	15		69.2	3.06	+85.0
Propanol 32%	10 ml/kg.	10	15		112.4	6.38	+201.5
Morphine SO <sub>4</sub>	1 mg/kg.	10	15		47.6	3.87	+27.3
Glucose	4 gm/kg.	10	30		48.1	2.31	+28.6
Glucose + epinephrine	4 gm/kg.		20		46.7	4.19	+24.1
	1 mg/kg.	10	10				
Post. pit X	20 mg/kg.	17	15		61.1	3.64	+66.7
Chloralose	100 mg/kg.	10	15		39.3	2.14	+5.1
Histamine	3 mg/kg.	9	10		32.0	1.28	-14.4
<i>Controls B</i>		35			28.07	1.35	
Prostigmine methyl SO <sub>4</sub>	0.4 mg/kg.	10	5		18.9	2.43	-32.7
Ephedrine HCl	8 mg/kg.	10	15		18.7	1.29	-33.4
Lobeline SO <sub>4</sub>	8 mg/kg.	9	10		22.6	1.49	-19.6
Epinephrine	1 mg/kg.	10	30		53.1	4.42	+88.9
Iodoacetic acid	40 mg/kg.	10	20		42.8	2.14	+52.3
Insulin	40 U/kg.	12	30		21.3	1.04	-24.2
Ergotoxine ethanesulfonate	10 mg/kg.	10	20		25.7	.715	-8.5
Desoxycorticosterone acetate	10 mg/kg.	11	30		30.6	1.56	+8.9

$$^1SE = \sqrt{\frac{\Sigma \Delta^2}{n(n-1)}}$$

37.4 seconds and *B* 28.07 seconds at 105 mm. Hg pressure. *Strain A* were mice of the Rockland strain; *strain B* were mice of the Purdue Hygienic strain.

The greatest effects on survival from explosive decompression are produced by the compounds which act as central depressants such as alcohols, or by chemicals which have hyperglycemic action such as epinephrine, posterior pituitary extract or glucose. Iodoacetic acid may belong to this group by preventing glycolysis. Substances causing central excitation such as ephedrine and lobeline tend to reduce survival time. So does insulin possibly by reducing blood sugar level. Other com-

pounds reducing survival time are prostigmine, histamine and ergotoxine. Desoxycorticosterone produces a slight increase in survival.

These results agree in general with previous observations (5-8) with survival of the isolated head, the survival of which was prolonged by glycotropic substances such as adrenaline, posterior lobe extract and glucose, but shortened by insulin and iodoacetic acid. Iodoacetic acid appears to behave differently in the present experiment. Previously iodoacetic acid has been reported to shorten survival in the isolated head (5) as well as in the entire animal (9). Hyperglycemia was reported to increase anoxic survival of the infant rat (1, 2, 5-10) but not of the adult (9). Hypoglycemia shortens survival of the young as well as of the adult (9). Morphine, alcohol and chloralose have been reported (7) to increase survival of the isolated head which agrees with findings in the present communication. In a report of the effects of autonomic drugs on anoxic survival (11) it was stated that ephedrine increased while lobeline and epinephrine decreased mortality to decompression. Our results agree as to ephedrine and epinephrine but not with lobeline. Our dose of lobeline was four times as great which might account for the difference. The posterior lobe extract was prepared by dissolving the dried gland in water and then filtering. The same method of preparation was used earlier (5) and the resulting product greatly prolonged the duration of gasping in the isolated head of the young rat. The extract undoubtedly contains the vasopressor principle of the posterior pituitary. It has been reported elsewhere (12) that the pressor principle elevates blood sugar and prolongs survival. Ergotoxine was suspected of possibly reversing the effect of epinephrine on survival. Chloralose has been reported (13) to increase the effectiveness of the reflex carotid centers which in themselves are necessary for gasping of the isolated head (14). It has been reported (11) that ergotoxine decreases and prostigmine greatly increases mortality to anoxia. We have found no reports of the effect of histamine on anoxic resistance.

#### SUMMARY

The effects of 16 chemical compounds on survival to explosive decompression at a pressure of 105 mm. Hg (equivalent to 3% oxygen at sea level pressure) were measured with mice, this pressure in the case of the mouse being equivalent to complete anoxia. Substances prolonging survival were the alcohols, glucose, morphine, posterior pituitary extract, epinephrine, iodoacetic acid, chloralose and desoxycorticosterone. Those shortening survival were ephedrine, insulin, histamine, lobeline, ergotoxine and prostigmine. The results are compared with those obtained by slow decompression and with the survival of the isolated head.

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# PROPERTIES OF THE TRANSSULFURASE RESPONSIBLE FOR CONVERSION OF CYANIDE TO THIOCYANATE

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WE HAVE recently shown that the enzyme responsible for the conversion of cyanide to thiocyanate is widely distributed in the animal body (1). Other workers have also described the distribution of this enzyme and have studied the *in vitro* conditions of the reaction. Konrad Lang, who first postulated the existence of an enzyme for the conversion of cyanide to thiocyanate in the presence of thiosulfate or colloidal sulfur (2), concluded that the reaction proceeded anaerobically according to the following equation:



He found that the optimal pH and substrate concentration for this reaction was 8.3 and 1 mole cyanide to 3 moles of thiosulfate respectively, and that the rate of reaction increased with increasing temperature up to 38°C. He used, however, a 1:1 ratio of cyanide and thiosulfate (0.005 M) at 20°C. for most of his studies. He also noted that the reaction proceeded according to the Schütz rule<sup>1</sup> and was inhibited by the bivalent cations of calcium and magnesium.

Cosby and Sumner (3) studied the activity of a purified acetone powder at pH 7.4, at 20°C. with substrate concentrations of .004 M potassium cyanide. These authors found that regardless of the concentration of cyanide employed, the reaction did not follow the Schütz rule. Rosenthal (4) studied the activity of water homogenates and acetone powders of various tissues at 20°C. in a phosphate buffer at pH 7.3, the substrate concentration being 0.05 M. Recently Bénard *et al.* (5, 6) have described a principle necessary for 'the anticyanide action of thiosulfate' which is probably identical with the enzyme studied here. They measured activity indirectly by studying the oxygen consumption of yeast emulsion in the presence of the principle, thiosulfate and cyanide, and obtained an optimal pH of 7.0.

Since the workers mentioned above have dealt in the main with acetone powders at 20°C. and at varying pH values and substrate concentrations, it was felt that a systematic study of a standardized system in which one factor at a time could be varied might yield information that would throw further light on the functions of this enzyme.

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<sup>1</sup>Schütz rule states:  $K = X\sqrt{N_e/t}$  where  $N_e$  is concentration of enzyme,  $X$  is amount of substrate that is transformed in time  $t$  and  $k$  is a constant.

## PROCEDURE

Homogenates of brain, kidney, and liver of rat, rabbit, dog and monkey were chosen for this study because of their high enzymatic activity. The method of preparing homogenates was the same as that reported previously (1). The standard system used was (M/15) phosphate buffer (pH 7.4), 0.42 M sodium thiosulfate, 0.3 ml. tissue homogenate, 0.14 M potassium cyanide in a total volume of 9.2 ml. added in the order named, and shaken for 15 minutes at 37.5°C. These conditions were determined earlier to be reasonably near the optimum for all tissues. The enzyme activity was estimated by measuring the thiocyanate formed as previously described (1). The conditions in this system were varied as described below to permit the determination of optimal conditions of pH, time, temperature and substrate concentration. The same enzyme preparation was used for each study of the effect of a variation. For all variations, blank determinations were made.

**pH.** Fourteen solutions of phosphate buffer of the same molarity (M/15) ranging in pH volume from 6.41 to 8.15 were used as the phosphate buffer of the standard system to determine the relation between the activity of various homogenates and the pH of the buffer. pH determinations on the complete system were made before and after incubation at 37.5°C. for 15 minutes.

**Time and Temperature.** Tissue homogenates were allowed to react in the standard system at 37.5°C. for varying intervals of time ranging from 1 to 50 minutes, and for 15 minutes at temperatures ranging from 1° to 58°C.

**Substrate Concentration.** The influence of substrate concentration was determined by varying the ratio of cyanide and thiosulfate from 1 to 6 using final cyanide concentrations of 0.05, 0.09, 0.14, 0.19 and 0.24 M.

The effects of sulfur-containing compounds, bivalent cations, iodoacetic acid, potassium fluoride and of cyanide were determined by adding known concentrations of these compounds to the standard system. The inhibiting compounds were added to the system immediately after the addition of the tissue extract and just before the addition of cyanide. However, when the inhibitory influence of cyanide itself was studied it was necessary to add the cyanide to the system before the addition of thiosulfate, since it has been shown that thiosulfate protects the enzyme against inhibition by cyanide (7).

The stability of the enzyme in intact tissue was studied by comparing the enzymatic activity of a 1:10 homogenate of half of a dog brain kept at room temperature for 6 hours before homogenization with that of a 1:10 homogenate prepared from the other half of the brain immediately after the animal was killed.

## RESULTS

**pH.** Figure 1 shows the variation of enzymatic activity with pH. The solid line represents a plot of the pH of the buffers added, while the broken line shows the pH of the complete system. At the homogenate dilutions and in the pH range reported here there is no measurable pH change during the course of the reaction. The optimum pH of the reaction appears to be approximately 9.1.

**Time and Temperature.** The activity of the homogenate increases with time of incubation in an almost linear fashion. The length of time required to reach maximum

activity appeared to be proportional to the enzyme concentration. The activity of the enzyme increases with temperature in a typical fashion, showing an optimum

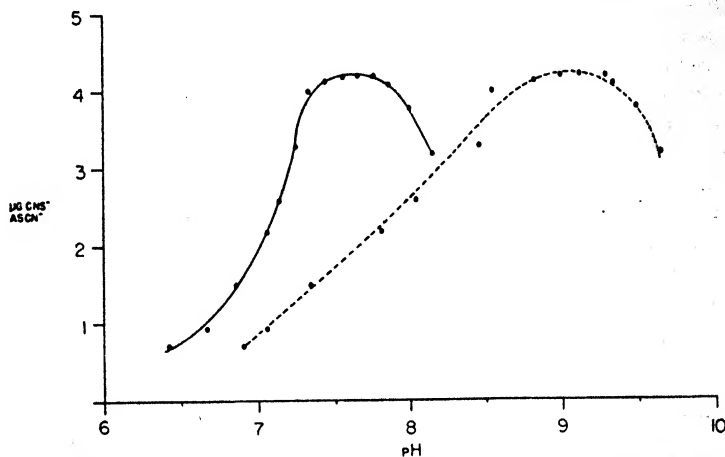


Fig. 1. VARIATION OF ENZYME ACTIVITY with pH of buffer and with pH of entire system. Solid line, pH of buffer; broken line, pH of entire system.

○, 0.05 M CN<sup>-</sup> ●, 0.09 M CN<sup>-</sup> □, 0.14 M CN<sup>-</sup> ■, 0.19 M CN<sup>-</sup> △, 0.24 M CN<sup>-</sup>

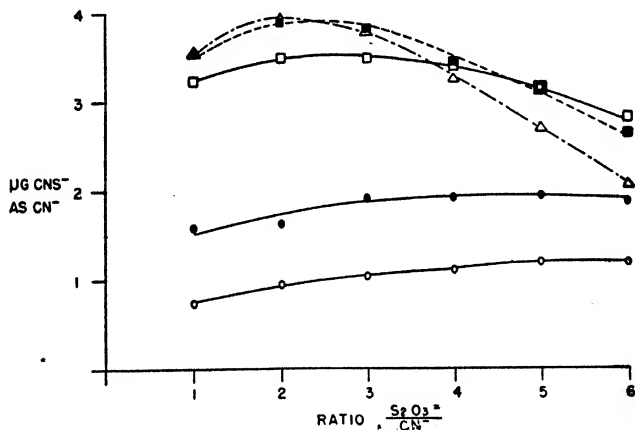


Fig. 2. VARIATION OF ENZYME ACTIVITY with ratio of  $S_2O_3^{2-}$  to  $CN^-$  using various  $CN^-$  concentrations.

activity in the neighborhood of 38° to 40°C., and dropping sharply thereafter to little or no activity at 58°C.

**Substrate Concentration.** Thiocyanate production with varying molar ratios of thiosulfate and cyanide depends on the molar concentration of the cyanide added. Thus in figure 2 it can be seen that when low cyanide concentrations are used, the

TABLE 1. EFFECTS OF ADDING CERTAIN DIVALENT CATIONS TO THE STANDARD ENZYME SYSTEM USED TO CONVERT CYANIDE TO THIOCYANATE

CATION <sup>1</sup>	FINAL CONC., M	% OF NORMAL VALUE <sup>2</sup>
Cu <sup>++</sup>	0.001	30
Fe <sup>++</sup>		66
Mn <sup>++</sup>		96
Si <sup>++</sup>		96
Ca <sup>++</sup>	0.01	105
Li <sup>++</sup>		110
Mg <sup>++</sup>		103
Ba <sup>++</sup>		110

<sup>1</sup> Added as the chloride.    <sup>2</sup> Value obtained in standard enzyme system.TABLE 2. INHIBITION OF THE FORMATION OF THIOCYANATE FROM CYANIDE BY CYANIDE ION<sup>1</sup>

CONC. KCN ADDED	AMOUNT ADDED	% INHIBITION <sup>2</sup>
M	ml.	
0.434	0.1	100
0.01	0.1	80
0.01 plus 0.3 ml sat. soln. cystine	0.1	0
0.001	0.1	45

<sup>1</sup> Added before thiosulfate.    <sup>2</sup> Based on comparison with activity of the standard system.

TABLE 3. EFFECT OF VARIOUS SULFUR-CONTAINING COMPOUNDS AND ENZYMIC INHIBITORS UPON THE ACTIVITY OF THE STANDARD ENZYME SYSTEM

AGENT	CONC.	AMOUNT ADDED	% OF NORMAL VALUE <sup>1</sup>
	M	ml.	
Cystine	0.42 <sup>2</sup>	0.9	118
Cystine	Sat	0.3	119
Diphenyl sulfide	0.42	0.9	110
Sodium sulfide	0.004	0.3	52
Sodium sulfide	0.42	0.9	35
Cysteine	0.42	0.9	17
Dithiobiuret	0.42	0.9	39
Sodium fluoride	0.01 M <sup>3</sup>		94
Sodium fluoride	0.02 M <sup>3</sup>		84
Potassium iodide	0.001 M <sup>3</sup>		94
Potassium iodide	0.002 M <sup>3</sup>		93
Iodoacetic acid	0.01 M	0.93	34
Iodoacetic acid plus 1 cc. 0.01 M cysteine	0.01 M	0.93	101
Iodoacetic acid plus 0.3 cc. sat. soln. cystine	0.01 M	0.93	43

<sup>1</sup> Value obtained in standard enzyme system.    <sup>2</sup> Since cystine is relatively insoluble a suspension was used.    <sup>3</sup> Final concentration.

highest activity is obtained with ratios of 5 and 6, and as the cyanide concentration is increased, the ratio for the highest activity is 2 or 3. In our standard system the



cyanide concentration is 0.14 M and the ratio of thiosulfate to cyanide is 3. This is close to the optimal ratio for this cyanide concentration.

Divalent cations, which have been reported by Lang (2) to reduce enzymatic activity, were found under our conditions to vary in their effects (table 1). Copper and iron produced significant inhibition, but the other cations tested had little or no effect.

The enzyme unless protected by thiosulfate is markedly inhibited by cyanide (7). The addition to the homogenate of 0.434 M KCN (final concentration 0.047 M)

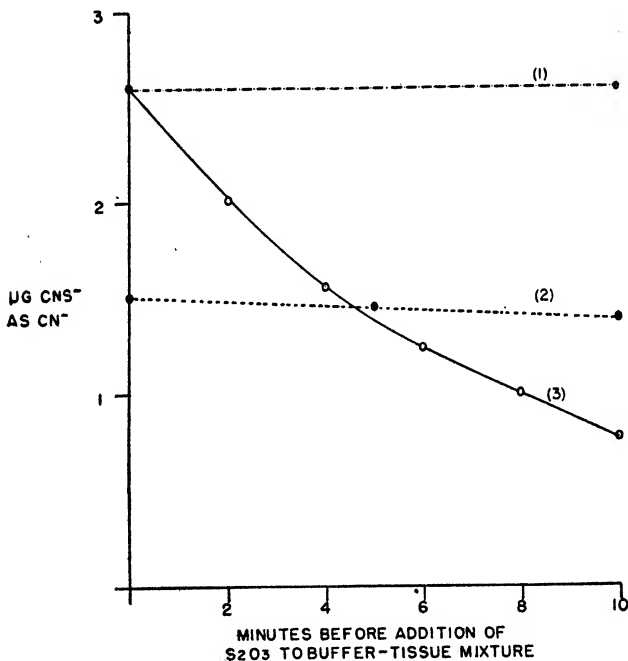


Fig. 3. DECREASE IN ENZYME ACTIVITY of dilute homogenates on standing with phosphate buffer at room temperature. Curve 1, activity on standing with  $S_2O_3^{2-}$ ; curve 2, activity when standing with cysteine; curve 3, activity when standing with phosphate buffer alone.

before adding thiosulfate results in complete inhibition of the enzyme. Cyanide inhibition can be prevented by the addition of cysteine immediately after the addition of cyanide (table 2). Iodoacetic acid also inhibits the enzymatic conversion of cyanide to thiocyanate but this effect can be completely prevented by cysteine, but not by cystine or methionine (table 3).

In studying the ability of sulfur compounds to replace thiosulfate (1), it was noted that certain of the compounds had a marked inhibitory effect on the enzymatic activity when added to the complete system. The most marked inhibition was noted with cysteine, sodium sulfide and dithiobiuret. Cystine, however, increased the enzymatic conversion about 18 per cent (table 3).

The enzyme when present in 1:10 homogenates of tissues showed a remarkable

stability. If, however, the homogenate was greatly diluted the enzyme became very unstable. When 0.3 cc. of a 1:10 homogenate were added to 7.3 cc. phosphate buffer (pH 7.4) and allowed to stand at room temperature the activity decreased rapidly. Loss of activity could be prevented either by the addition of cysteine or of thiosulfate (fig. 3).

#### DISCUSSION

It is evident that the phosphate solution used here did not sufficiently buffer the system to counteract the strong alkaline effect of potassium cyanide. Lang (2), reported a sharp pH optimum of 8.3 for his system, but this was the pH of the buffer and he did not give the pH of the complete system. Homogenates prepared from rabbit liver showed an even higher optimal pH than those prepared from tissues of other species and from other rabbit tissues.

The inhibition with iodoacetic acid and its reversal by cysteine indicates that the removal of sulphydryl groups at least partially inactivates the enzyme. The marked sensitivity to cyanide suggests that the enzyme contains a heavy metal group which is necessary for its activity. We have interpreted the data on the protective action of thiosulfate against cyanide to mean that the enzyme and thiosulfate form a loose combination possibly through sulfur which breaks down in such a way that sulfur is transferred from thiosulfate to an acceptor, in this case, cyanide.<sup>2</sup> The inhibitory effect of certain sulfur compounds suggests that they block the enzyme so that it cannot combine with thiosulfate. We cannot explain the accelerating effect of cysteine unless we consider that some cyanide inactivation goes on in the system even in the presence of thiosulfate and that cystine protects against this.

#### SUMMARY

The transsulfurase responsible for the conversion of cyanide to thiocyanate in the presence of thiosulfate has an optimal pH of 9.1 and a temperature optimum of 38° to 40° C. In highly active preparations the amount of thiocyanate formed is a linear function of time. At a final cyanide concentration of 0.14 M the most effective ratio of cyanide to thiosulfate is 1:3.

Data obtained using inhibitors indicate that sulphydryl groups are partly responsible for the activity and that the enzyme contains a heavy metal. It is suggested that the enzyme forms a loose combination with thiosulfate which breaks down to yield sulfur in a form which can be accepted by the cyanide ion.

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<sup>2</sup>The ability of thiosulfate to protect against oxidation may be in part due to its mild reducing properties.

# CHANGES IN PLASMA OF THE RAT DURING FASTING AND INFLUENCE OF GENETIC FACTORS UPON SUGAR AND CHOLESTEROL LEVELS<sup>1</sup>

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THAT the appetite varies in health and disease and is sensitive to many experimental procedures is well known. It was therefore of interest to determine the effects of fasting upon the composition of the blood plasma of the rat and to seek conditions which would control this unwanted source of variation. We particularly felt the need of such data as a prerequisite to the examination of the plasma of animals subjected to total body irradiation with x-rays, a procedure causing a marked decrease in food consumption. It is known, of course, that the plasma level of glucose falls during starvation, but the complexities of its course during a week of fasting are not generally known, nor, as will be shown, the parallel changes in the levels of nonprotein nitrogen (NPN) and total protein. A different pattern of change was found in the levels of hemoglobin, albumin-globulin ratio (A/G), and chloride. No change occurred in the cholesterol level.

It was realized that the composition of the plasma, and the pattern of change during fasting, might depend upon the strain of animals employed; 4 strains were therefore examined. Genetic differences were found which independently affected the plasma levels of glucose and cholesterol, but not of the other components. The differences in cholesterol level were abolished by hypophysectomy. As to the pattern of change induced by fasting, this was alike in all strains excepting one, where the course of the glucose level differed from the rest. The atypical inheritance of this difference is described.

## METHODS

The strains of rats used were the Sprague-Dawley (SD) and Holtzman (H) obtained from commercial breeders at Madison, Wisconsin, and the Tumblebrook hooded (TBH) obtained from Tumblebrook Farm, Brant Lake, New York. The

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animals were bought at the age of 30 days and raised on Purina Laboratory Chow checkers. The fourth strain, obtained through the kindness of Mrs. D. Beard of the Duke Medical School, was the Vanderbilt Osborne-Mendel (OM) and was bred in this laboratory. The nongenetic experiments were on mature animals, at least 90 days old, the females weighing more than 170 gm., the males more than 225 gm. The animals were individually caged and numbered 2 to 5 days before an experiment began. During the periods of fasting, water was always available.

With the exception of some of the original parents, the animals employed in the genetic experiments had spent their entire lives in the air-conditioned animal rooms, maintained at 70° F. and 50 per cent relative humidity. After weaning, they were fed Purina Laboratory Chow checkers and were kept singly or in pairs in wire-mesh cages. The principal crosses were as follows:

*SD* × *TBH*. One *SD* male was mated with 5 *TBH* females and 5 *F*<sub>1</sub> litters were obtained from which 28 animals were tested. Pairs of litter mates from 4 of the 5 litters were crossed and 9 *F*<sub>2</sub> litters were obtained. From 8 of the *F*<sub>2</sub> litters, 54 animals were taken for the blood tests.

*OM* × *TBH*. Two *TBH* males were crossed with 5 *OM* females, producing 5 litters and one *OM* male was crossed with 4 *TBH* females, producing 4 litters, or 9 *F*<sub>1</sub> litters in all, from which 52 animals were tested. Pairs of littermates from 2 of these litters were crossed, and produced 6 *F*<sub>2</sub> litters. Nonsibling crosses were made between 3 males and 15 females from the other 7 litters, making a total of 21 *F*<sub>2</sub> litters. For the blood tests, 74 animals were taken from 17 of the *F*<sub>2</sub> litters.

*OM* × *H*. One *H* male was crossed with 5 *OM* females, producing 5 *F*<sub>1</sub> litters.

*SD* × *OM*. Each of 2 *SD* males was mated with 4 *OM* females, producing 8 *F*<sub>1</sub> litters.

All of the *F*<sub>1</sub>'s, or the *F*<sub>2</sub>'s, of each cross were within one or 2 weeks of the same age, and were tested for their sugar and cholesterol levels at the same time, when their weights ranged from 150 to 200 gm. Blood was taken by cardiac puncture under ether anesthesia with heparin as anticoagulant. Reasons for the choice of this procedure, together with the methods of chemical analysis, were detailed previously (1). It was concluded that normal values were obtained, unaffected by the technique of sampling, in all tests except that for glucose. The latter was about 15 mg. per cent too high, due to the anesthesia and to the use of a tungstic acid filtrate (instead of a zinc hydroxide one). All determinations were made on plasma with the exception of that for hemoglobin. In the nongenetic experiments no animal was ever bled a second time.

The statistical definitions followed Snedecor (2). Unless specified otherwise, groups of animals are described in terms of the mean and standard deviation of the series (not standard error), followed by the number of individuals in brackets. The range encompassed by the mean plus and minus the standard deviation includes two-thirds of the observations.

#### EXPERIMENTAL RESULTS

A 24-hour fast is frequently employed before taking blood samples. Table 1 shows the analysis of plasma for the 4 strains of rats under this condition. While

in most cases the agreement of the means and of the standard deviations was striking, significant strain differences were noted in the sugar and in the cholesterol levels. To estimate further the effect of fasting, other experiments were made in each of which about 20 animals were used. Food was withdrawn from the cages on day 0 at 9 A.M., and on that day and various days thereafter 2 to 4 animals were bled at 9 A.M. and discarded. The data from the 4 strains were sufficiently alike to be pooled, except for the TBH hemoglobin and sugar (fig. 1). During the first 4 or 5 days, chloride and A/G changed very little, though both rose thereafter. The glucose, protein and NPN levels showed parallel changes which oscillated about the 24-hour fasting value. The level of the hemoglobin fell at first, but rose steadily thereafter. The cholesterol level, as shown previously (3), was not affected by fasting and was therefore not graphed. The sugar level in strain TBH was about 15 mg. per cent lower than in the other strains on *day 0*, and since it did not rise again on *days 3* or *4*, the difference increased to 50 mg. per cent (fig. 1, legend).

TABLE 1. COMPOSITION OF PLASMA AFTER 24 HOURS OF FASTING<sup>1</sup>

STRAIN	TBH	H	SD	OM
Sugar (mg. %)	129±4[27] <sup>2</sup>	144±7[25]	140±9[43]	143±5[28]
NPN (mg. %)	32.7±2.8 [25]	32.6±1.2 [25]	32.4±1.4 [43]	32.3±.9 [32]
A/G	2.01±.04 [26]	2.02±.04 [24]	2.0±.01 [17]	2.02±.04[29]
Protein (gm. %)	6.72±.12 [27]	6.79±.14 [24]	6.67±.19 [38]	6.77±.12 [31]
Chloride (mg. %)	608±7 [49]	607±7 [39]	606±10 [55]	609±7 [35]
Cholesterol (mg. %)	68.5±3.3 [20]	65.3±2.8 [16]	120±6 [39]	132±4 [10]

<sup>1</sup> Water available. <sup>2</sup> Mean and standard deviation for the bracketed number of animals.

For comparison with table 1, the following pooled *day 0* (nonfasting) values are given: glucose (TBH strain omitted), 169±3 mg. per cent (15); NPN, 41.3±.6 mg. per cent (19); protein, 7.1±.12 gm. per cent (20); A/G, 2.01±.04 (20); chloride, 609±7 mg. per cent (21).

To estimate how soon the normal plasma levels reappeared after a period of fasting, pairs of SD animals were starved for 1, 2, 3, 4 and 5 days. In one series, analyses were made after 2 days of refeeding, in a second series after 5 days of refeeding. The chloride levels were normal throughout. In the case of NPN, glucose and protein, fasting for one day required 2 to 3 days for recovery; fasting for 2 to 3 days required about 5 days for recovery. A 5-day recovery period brought the NPN and glucose to normal after a 5-day fast, but the protein was slow by about 0.3 gm. per cent. In no case was there evidence of overshooting the normal values.

The striking differences in the cholesterol levels, shown in table 1, were examined genetically by a number of crosses, with the following results in mg. per cent:

SD × TBH: F<sub>1</sub>, 88.6±4.7 (25); F<sub>2</sub>, 88±14 (50).  
 OM × TBH: F<sub>1</sub>, 93.3±5.5 (42); F<sub>2</sub>, 92.6±13 (64).  
 OM × H: F<sub>1</sub>, 93.8±3.2 (16).  
 OM × SD: F<sub>1</sub>, 123.2±3.9 (5 from 5 litters).

The data show that crossing any 2 strains (table 1) led to an  $F_1$  of intermediate value. In the case of the 'high' (OM, SD)  $\times$  'low' (H, TBH) crosses, the  $F_1$ 's were not the exact arithmetic mean of their parents (P generations), being 5 to 8 per cent too low. The standard deviations of the P and of the  $F_1$  generations were about the same.

When the  $F_1$ 's were inbred, the means of the resulting  $F_2$ 's agreed with those of their respective  $F_1$ 's within one per cent, but the standard deviation was about three times as large. Frequency distribution plots of the  $F_2$  values approximated the normal curve, and the extreme values reached but did not exceed those of the grandparents (SD or OM or TBH). The data were typical of the case in which a number of genes are involved (4).

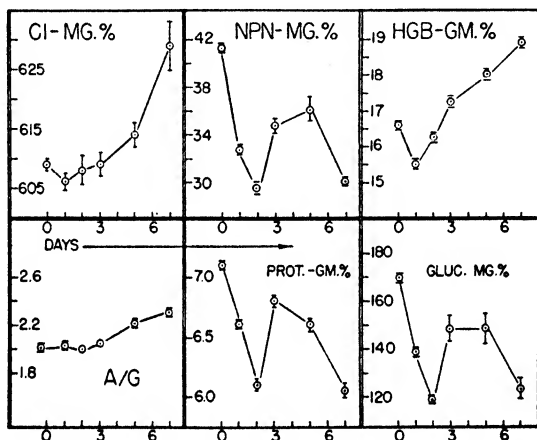


Fig. 1. EFFECT OF STARVATION. Standard errors are indicated when they are larger than the plotted symbol. Each point is the mean of 14 to 20 different animals, from 6 experiments (2 SD, 2 OM, 1 H and 1 TBH). Some data for the TBH strain were not averaged; TBH hemoglobin paralleled but rose more quickly than the rest; TBH glucose was 153 mg. per cent on day 0, 94 on day 2, and 87 on day 5.

It was of interest that the genes under discussion shifted the cholesterol level only within a certain range, suggesting that they affected some regulatory mechanism rather than that directly responsible for the synthesis of cholesterol. Two regulators are known in the rat, the thyroid (5) and the pituitary gland (6), the removal of either causing a marked rise in plasma cholesterol. 'High' and 'low' strains were therefore compared after hypophysectomy. Thirty days following operation, the levels for the H and SD strains, originally 65 and 120 mg. per cent, respectively, were  $188 \pm 8$  (9) and  $192 \pm 5$  (14) mg. per cent. This loss of phenotypic difference following hypophysectomy suggested that the genes might operate by way of the pituitary or one of its target organs.

Analysis of the sugar data involved a consideration of two factors, the concentration of the plasma glucose on any given day of fasting, and the magnitude of the rise in glucose occurring between the second and third days of fasting. Table 1 showed

the TBH glucose level to be significantly below those of the 3 'high' strains after a 24-hour fast, and table 2 showed the same to be true for *day 0* and especially *day 3*. Likewise, the third-day increment was prominent in the 'high' strains, but negligible in the TBH strain; and the fourth- and fifth-day levels, which continued elevated in the other strains, fell 5 to 10 mg. per cent in the TBH strain. The data for *days 1, 2* and *4* were omitted from table 2 to shorten it, since in all cases they were consistent with those exhibited.

When the TBH strain was crossed with the OM or the SD, the mean of the  $F_1$  generation was equal to the arithmetic mean of the parents, and the standard deviations of the  $F_1$ 's and the P's were about equal (table 2). This was true both for the plasma level of glucose and for the third-day increment. Up to this point, the behavior of the glucose levels was like that of the cholesterol.

TABLE 2. PLASMA GLUCOSE DURING FASTING IN VARIOUS CROSSES<sup>1</sup>

TYPE		DAY 0	DAY 3	DAY 3-DAY 2
Cross	Group	mg. %	mg. %	mg. %
High $\times$ High <sup>2</sup>		170 $\pm$ 3.7 [32]	154 $\pm$ 3.6 [29]	35.8 [28] <sup>3</sup>
TBH $\times$ TBH		152 $\pm$ 2.9 [15]	94.9 $\pm$ 3.2 [21]	-0.5 $\pm$ 2.4 [19]
OM $\times$ TBH, $F_1$			123 $\pm$ 2.7 [29]	15.1 $\pm$ 3.3 [26]
OM $\times$ TBH, $F_2$	1	153 $\pm$ 4 [25]	96.9 $\pm$ 4.3 [24]	2.1 $\pm$ 3.4 [20]
OM $\times$ TBH, $F_2$	2	170 $\pm$ 3.8 [40]	148 $\pm$ 7.1 [37]	28 $\pm$ 3 [34]
OM $\times$ TBH, $F_2$	1 + 2	163 $\pm$ 9.1 [65]	128 $\pm$ 30 [61]	18.4 $\pm$ 13 [54]
SD $\times$ TBH, $F_1$		158 $\pm$ 2.5 [6]	122 $\pm$ 2.1 [10]	16.3 $\pm$ 3.4 [10]
SD $\times$ TBH, $F_2$	1	156 $\pm$ 5 [29]	104 $\pm$ 9 [26]	2.8 $\pm$ 2.7 [27]
SD $\times$ TBH, $F_2$	2	171 $\pm$ 4.3 [21]	148 $\pm$ 10.5 [16]	29.8 $\pm$ 7.1 [16]
SD $\times$ TBH, $F_2$	1 + 2	163 $\pm$ 7.9 [50]	120 $\pm$ 23 [42]	14 $\pm$ 13 [43]

<sup>1</sup> Mean and standard deviation for the bracketed number of animals. <sup>2</sup> SD  $\times$  SD, OM  $\times$  OM, H  $\times$  H, H  $\times$  OM, SD  $\times$  OM. <sup>3</sup> Difference of the means of *day 3* and *day 2*. The mean of the individual differences of the 14 animals done on both days was 34.3 $\pm$ 2.7.

When the  $F_1$  generations were inbred, the results partly depended upon the cross involved. The  $F_2$  progeny of the OM  $\times$  TBH cross was distributed into two classes, which fell on either side of the  $F_1$  population (fig. 2). The lower class, containing 37 per cent of the progeny, was like the TBH grandparent, both in regard to plasma-sugar level and third-day increment (table 2). The other class, containing about 63 per cent of the progeny, had values which were slightly lower than their 'high' type OM grandparent.

The  $F_2$  progeny of the SD  $\times$  TBH cross likewise fell on either side of the  $F_1$  population (table 2, fig. 3) but the separation was not so marked as in the previous cross. The lower group was like its TBH grandparents. The other formed a more or less continuously varying population in which the magnitude of the third-day increment was a function of the third-day plasma-sugar level.

It was remarkable that the  $F_2$  glucose data did not appreciably overlap the range of the  $F_1$  data, in marked contrast to the results for cholesterol. Whatever the basis of this puzzling result may have been, the statistics on litter size indicated

that it did not involve selection by death *in utero*. In the case of the OM  $\times$  TBH crosses, the  $F_1$  litters averaged  $7.6 \pm 2.6$  (9) animals and the  $F_2$ 's,  $8.7 \pm 2.1$  (21). In the case of the SD  $\times$  TBH crosses, the  $F_1$ 's averaged  $7.2 \pm 0.9$  (5) animals, and the  $F_2$ 's,  $7.8 \pm 3.7$  (9). Since these litters were the first to be thrown by the females involved, they were somewhat small in size. It was our impression that they averaged about the same as for the SD or OM strains, and were somewhat larger than had been obtained from the TBH strain where 6 was about the usual number.

That inadvertent selection of the tested animals had not occurred was indicated at least to the following extent. 1) Cholesterol and sugar analyses were made on the same samples of blood, but gave a different genetic result. 2) Records were kept of the inheritance of albinism, coat color and the pattern of coat color. These be-

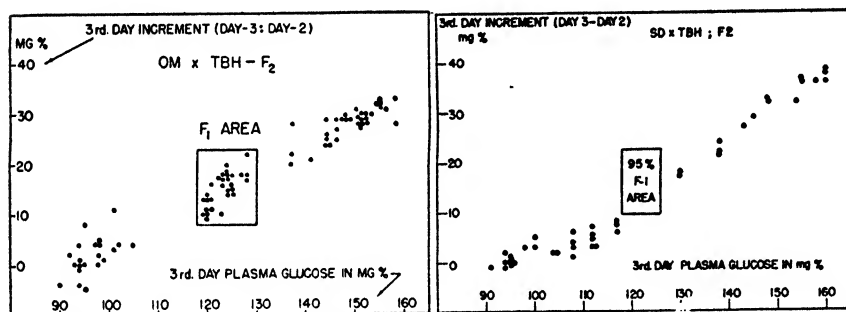


Fig. 2. (left). THIRD-DAY INCREMENT IN SUGAR as a function of third-day plasma level for OM  $\times$  TBH cross. The results for the  $F_1$  generation fell in the boxed-off area. The  $F_2$  data fell into two groups, on either side of the  $F_1$  data. The lower group resembled the TBH grandparent, the upper resembled the OM grandparent.

Fig. 3 (right). THIRD-DAY INCREMENT IN SUGAR as a function of third-day plasma level for SD  $\times$  TBH cross. The area is shown in which 95 per cent of the  $F_1$  observations fell, based on the  $F_1$  means and standard deviations for 10 animals. The  $F_2$  data fell into two groups on either side of the  $F_1$  data, each with a different slope.

haved as indicated by previous workers (7). 3) No prominent evidence of linkage with respect to sex, the nature of the coat or cholesterol level was noted. 4) The animals employed in each test were selected in such a way that approximately the same percentage of each of a number of litters was taken (see METHODS).

#### DISCUSSION

Figure 1 shows that during the first 4 to 5 days of fasting the changes in chloride and A/G were very small, and during the first week the levels of NPN, sugar and protein tended to oscillate about the 24-hour fasting level. Although paired-feeding is the ideal control in experiments where appetite may be affected, the present results indicate that 24-hour starved controls may prove quite adequate in short experiments.

The course of the changes in figure 1 is difficult to explain. While dehydration may have played a role, the fall in NPN, protein and glucose at a time when the chloride and hemoglobin levels were rising showed that other important factors



were at work. The parallel courses of the NPN, protein and glucose levels suggested that they might be under a common control, but specific information is lacking.

That the level of cholesterol did not change during fasting had been reported by Sure *et al.* (3) in their strain of rats. However, Levin (8) found a 28 per cent decrease in the adult Long-Evans male after 3 days of fasting. It is of interest that this strain was also found to differ from the SD and H strains with respect to the changes in the plasma following hypophysectomy (unpublished data).

Selye (9) noted a variable rise in venous blood sugar on the second or third day of fasting in 6 adult and 2 of 4 immature rats. Handler and Georgeaide (personal communication) found a sugar curve similar to that in figure 1, and have shown that the third-day increment depends upon an adequate protein level in the diet prior to fasting. They have obtained other evidence that this effect of diet may be mediated through the pituitary gland rather than through the decrease in protein reserve available for gluconeogenesis. The adrenal shows a slight rise in weight after a week of fasting, and arguments have been advanced attributing this to a change in pituitary function (10). In the mouse, a small rise in blood sugar occurs on the third day of fasting. However, hereditary dwarf mice which lack the pituitary acidophil cells show an equal rise (11).

Comparable sets of day-by-day analyses during the first week of fasting are lacking for other species. The available data for man (12, 13), the dog (14, 15), and the guinea pig (16) do not indicate a parallel course for NPN, protein and glucose, nor the type of curve in figure 1. For the rat, additional data are available on the total amount of plasma protein after one or 2 weeks of fasting (17, 18).

The genetic data open the possibility for further experiments. There is evidence that hypercholesterolemia in man is an incomplete dominant trait (19), and it now may be possible to contribute to this problem by studies in the rat. The strain differences in the plasma level of glucose and its behavior during fasting presumably are indicative of other more fundamental differences in physiologic constitution, and may point the way to their investigation. Castle (7) has listed the 23 known genes for the rat. The genetic factors discussed above would seem to be more promising for physiological metabolic research than those previously reported.

#### SUMMARY

The composition of the plasma of the rat was examined during the course of one week of fasting. Data for the Osborne-Mendel, Holtzman and Sprague-Dawley strains agreed well enough to be pooled. During the first 4 to 5 days of fasting, the changes in chloride and albumin-globulin ratio were negligible, after which both rose moderately. During the entire week, the levels of sugar, nonprotein nitrogen and total protein oscillated systematically about the 24-hour fasting level. Hemoglobin fell about one gm. per cent during the first day, and thereafter rose at the rate of about 0.5 gm. per cent per day. Similar results were obtained with the Tumblebrook Farm hooded strain, except in the case of the sugar level which was somewhat lower than in the other strains, and which fell steadily during fasting. Breeding experiments established this difference to depend chiefly upon genetic characters. The plasma

cholesterol levels were not affected by fasting. In the Sprague-Dawley and Osborne-Mendel strains they were twice those in the Holtzman and Tumblebrook hooded strains. Breeding experiments showed that the genetic basis for this difference involved a number of genes. Following hypophysectomy, the cholesterol rose to a new level, which was the same in both the 'high' and 'low' genetic types.

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# EFFECT OF FEEDING AN 'ANIMAL PROTEIN FACTOR' CONCENTRATE TO YOUNG LAMBS

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AS EARLY as 1946, Bird *et al.* (1) and Whitson *et al.* (2) reported that the addition of cow manure to a basal soybean diet for hens improved hatchability and eliminated the usual seasonal variation in hatchability. Thus, they concluded, cow manure contains an unidentified factor which promotes good hatchability. More recently, Abelson and Darby (3) reported the isolation of vitamin B<sub>12</sub> from sheep feces and showed conclusively that sheep are able to synthesize this vitamin.

In spite of this evidence to show that ruminants are able to synthesize vitamin B<sub>12</sub>, it was decided to try to find if any benefits might be derived from adding an animal protein factor supplement (source of vitamin B<sub>12</sub> and aureomycin<sup>1</sup>) to the creep ration of lambs. It was felt that by supplying this additional source of vitamins, additional benefits, such as improved growth rate, might be encountered before the rumen flora was well developed.

## EXPERIMENTAL PROCEDURE

Fifty-one lambs of Rambouillet, Delaine Merino, and Dorset crossbred breeding were used in this study. The lambs ranged in weights from 7.5 to 68 pounds and ranged in age from 4 to 12 weeks with an average age of about 7 weeks. They were divided into two lots on the basis of random assortment. The lambs were weighed at the beginning of the trial and wool samples were collected from the right shoulder of each of the lambs. After 56 days, all lambs were weaned and were then continued on experiment for 28 more days. At the time the lambs were weaned, blood samples were collected for vitamin B<sub>12</sub> analysis.

The lambs were weighed after each 28 days and at the close of the experiment. At this time wool samples were collected from the left shoulder of each lamb, blood samples were collected for vitamin B<sub>12</sub> analysis by method of Skeggs *et al.* (4), and 4 lambs from each lot were slaughtered so the vitamin B<sub>12</sub> content of the liver, pancreas, kidney and spleen could be determined. The first group of lambs was fed a ration which consisted of oats 37 per cent, milo 37 per cent, bran 15 per cent, cottonseed meal 10 per cent, plus alfalfa hay, free choice. The lambs in lot II received the above ration plus 1 per cent of an A.P.F. supplement (containing .6 µg. vitamin

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<sup>1</sup> According to information obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., this sample of A.P.F. contained 4.0 mg. aureomycin per gm.

B<sub>12</sub>/gm. and 4.0 mg. aureomycin/gm.) for 2 weeks and 0.5 per cent of such supplement for the remainder of the trial. Vitamin B<sub>12</sub> content of the A.P.F. supplement was determined by the method of Skeggs *et al.* (4). All lambs had free access to a mixed grass pasture.

### RESULTS AND DISCUSSION

The results of the feeding test with the lambs before and after weaning are summarized in table 1. The A.P.F. supplement included in the ration of the lambs in *lot II* consisted of one per cent of the ration for the first 2-week period. During this time the lambs ate almost no grain supplement, due apparently to the A.P.F. present, so the A.P.F. content of the ration was accordingly lowered to 0.5 per cent of the ration for the remainder of the trial.

TABLE 1. GROWTH AND WOOL DATA OF LAMBS FED WITH AND WITHOUT A.P.F. SUPPLEMENT

Lot No.	I	II
Type supplement fed.....		A.P.F.
Av. initial wt.....	38.4	37.3
Av. weaning wt.....	65.4	62.6
Av. daily gain to weaning.....	.46	.45
Av. final wt.....	76.0	69.0
Av. daily gain after weaning.....	.38	.22
Av. initial fiber length, mm.....	19.36	18.67
Av. final fiber length, mm.....	40.70	38.10
Percentage increase.....	52.43	51.0
Av. initial fiber diameter, $\mu$ .....	15.61	15.02
Av. final diameter, $\mu$ .....	18.99	17.45
Percentage increase.....	3.41	2.41

It may be noted from table 1 that there was no difference in the average weight of the two lots of lambs at weaning time. Even when the ration contained only 0.5 per cent A.P.F., it seemed to be less palatable and the lambs in *lot II* consistently ate a little less feed than those in *lot I*. Apparently, the milk from the dams was sufficient to overcome the effect of this lowered feed consumption.

In the 28-day period following weaning, the differences in growth and feed consumption between the two lots of lambs became much more striking. The lambs in *lot II*, during the last 14 days of the period, almost entirely stopped eating their grain supplement, leaving them pasture as the only source of feed. Since these lambs were breeding animals and since they had almost entirely stopped eating the grain, which contained the A.P.F., the test was concluded. The average daily gain for the 28-day period following weaning was .38 pounds per day for the lambs in *lot I* and .22 pounds per day for the lambs in the A.P.F. supplemented lot (*lot II*).

The wool growth data for the two groups of lambs are shown in table 1. These indicate that the A.P.F. supplement had a slight depressing effect upon both the diameter and length of the wool fibers. These differences indicate a slight advantage for the non-supplemented group (*lot I*).

The data on the vitamin B<sub>12</sub> levels of the blood and tissue are shown in table 2. Blood samples were collected at both weaning time and at the close of the experiment. The order of magnitude of difference is the same at both times and shows that the A.P.F. supplement had a depressing effect upon the vitamin B<sub>12</sub> levels of the blood.

As shown in table 2 no differences were observed in the tissue levels of vitamin B<sub>12</sub> of the two groups of lambs. This is not unexpected since the test was of short duration. A longer test might have shown differences. Further, since the ruminant does synthesize vitamin B<sub>12</sub>, it is expected that there would be tissue storage of this vitamin and that some time would be required to produce changes. These data do, however, give an indication of the levels of vitamin B<sub>12</sub> that may be expected in the internal organs of lambs.

It is interesting to speculate on why the A.P.F. supplement has a harmful effect on young ruminants when it has been shown so many times to be beneficial for

TABLE 2. BLOOD AND TISSUE LEVELS OF VITAMIN B<sub>12</sub> OF LAMBS FED AN A.P.F. SUPPLEMENT

Lot No.	I	II
Supplement fed.....		A.P.F.
Av. B <sub>12</sub> levels of blood, mμg/ml.....		
Weaning time.....	.71	.49
Close of experiment.....	.84	.57
Av. final B <sub>12</sub> levels of tissue, μg/100 gm.....		
Liver.....	38.6	35.4
Spleen.....	18.6	18.7
Kidney.....	23.6	28.8
Pancreas.....	21.8	19.4

mono-gastric animals. Since the type A.P.F. supplement used contains aureomycin, it is probable that this antibiotic interferes with the vitamin synthesizing rumen bacteria, thereby producing harmful effects. This was more or less 'hidden' during the suckling period since the lambs had their mothers' milk to rely upon as a source of essential nutrients; even then, the vitamin B<sub>12</sub> levels of the blood had already been interfered with. After weaning time, when there was no longer a source of milk available, these differences became more apparent—especially as reflected by growth and behavior. It is felt that the decrease in weights of the lambs fed the A.P.F. supplements is due to the aureomycin contained in this supplement.

Other data which have been presented by Colby *et al.* (5) show that the feeding of 100 mg. aureomycin per lamb per day produced very detrimental effects on the lambs as regards loss of weight and decreased feed consumption and also produced a decided change in the microflora of the rumen. It is of further interest to note that Stokstad and Jukes (6) have recently reported aureomycin to be a growth promoting factor for chicks, in contrast to these findings reported with lambs.

#### SUMMARY

The feeding of an animal protein factor supplement to young lambs decreased growth and feed consumption during the post-suckling period. Due, probably, to the

protective effect of the maternal milk, growth of lambs fed an A.P.F. supplement was not affected during the suckling period, though feed consumption of this group was reduced. The A.P.F. supplemented lambs produced wool fibers of slightly shorter length and slightly smaller diameters than non A.P.F. supplemented lambs. The A.P.F. supplement produced significantly lower levels of vitamin B<sub>12</sub> in the blood of the lambs at both weaning time and during the feeding period following weaning. No differences were observed in the tissue levels of vitamin B<sub>12</sub> of the two groups of lambs.

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# EFFECT OF RENAL ARTERIAL CONSTRICTION ON EXCRETION OF SODIUM AND WATER<sup>1</sup>

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THE rate of sodium excretion appears to be dependent on the balance between filtration at the glomeruli and reabsorption by the tubules. It is also possible that sodium is actively secreted into the urine by the renal tubules but at the present time there is no convincing evidence that suggests such a process. If the rate of sodium reabsorption is essentially predetermined *a*) by the glomerular filtration rate, and *b*) by a relatively fixed transfer maximum in the distal segment of the tubule, as suggested by Wesson, Anslow and Smith (1), then the rate of filtration governs the rate of sodium excretion. However, it is equally possible that the reabsorptive rate of sodium is to some extent an independent variable and may thus in part regulate the excretion of sodium. Several groups of investigators have presented evidence that suggested such a conclusion. Shannon (2) has related increased rate of urine flow through the proximal segment of the tubule to reduction in proximal segment reabsorption of sodium. In his studies the increased rate of tubular flow resulted from an increase in filtration rate but it is possible that tubular flow may increase or decrease for other reasons. Mudge, Foulks and Gilman (3) have suggested that the rate of sodium reabsorption may be regulated in part by the concentration of sodium in the tubular urine. Several investigators (4-6) have pointed out that the diminished excretion of sodium in congestive heart failure may be related to tubular factors rather than reduction in filtration rate alone. Likewise, there is some evidence that muscular activity and/or the upright position may diminish the excretion of sodium by means other than a reduction in filtration rate (7). Roem-melt, Sartorius and Pitts (8) have conclusively demonstrated that a hormone or hormones of the adrenal cortex regulate the reabsorption of sodium independently of the rate of glomerular filtration. It may be mentioned that the decreased percentage of filtered sodium reabsorbed at high concentrations of sodium in the plasma (9, 10) may partly result from this adrenal cortical mechanism (8, 11). Also it is believed that the antidiuretic hormone of the posterior pituitary has an inhibitory effect on the reabsorption of sodium by the proximal segment of the tubule (2). In brief, not

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including the hormonal factors which might be considered as regulators of some sort of transfer maximum, two definite variables other than filtration rate have been suggested which could influence sodium excretion, viz. 1) the concentration of sodium in the plasma or tubular urine, and 2) the rate of urine flow through the tubule.

The present experiments are an attempt to elucidate further the role played by the filtration rate, as well as intrarenal mechanisms other than the filtration rate, in the control of sodium reabsorption and excretion in the dog.<sup>4</sup>

#### METHODS

The experimental conditions were set up to observe the influence of reduced blood flow on renal function in one kidney while the other kidney served as a control. Male and female dogs weighing 10 to 15 kg. were anesthetized with 30 to 35 mg/kg. sodium pentobarbital. The trachea and both carotid arteries were exposed and the trachea cannulated. The abdomen was opened by mid-line incision and both ureters cannulated with plastic tubing. The abdomen was then closed with the catheters exteriorized. The animal was placed on its left side and through a small incision just below the costal margin the right renal artery was exposed. A Goldblatt clamp was placed around the right renal artery as gently as possible and the wound edges were allowed to close.<sup>5</sup> An indwelling needle was inserted into one carotid artery and the other was cannulated for the purpose of measuring mean arterial pressure.

The procedure for an experiment was as follows. Collection of urine samples was started 45 minutes or more after positioning the clamp. The samples from the 2 kidneys were taken in periods of equal duration, lasting from 5 to 15 minutes depending on the rate of urine flow. The urine was collected directly into graduated cylinders of appropriate size. Blood samples were obtained from the carotid artery every 10 to 20 minutes throughout the course of the experiment. After two or three control urine-collection periods the edges of the flank incision were separated and the adjustable plate of the Goldblatt clamp was screwed in from 0.5 to 2.5 turns. Two to four periods of urine collection were carried out with the artery partially occluded. The clamp was then opened and urine collected for two or three recovery periods. In the initial experiments the partial occlusion of the artery was accomplished all at once with the result that the initial decline in urine flow was marked but tended to recover. In later experiments urine collection was discontinued for 5 to 10 minutes, during which time occlusion of the artery was carried out in step-wise fashion over a period of several minutes. When the desired decrease in urine flow was obtained, 3 to 5 minutes were allowed to elapse prior to resuming urine collection. This technique was also used when the clamp was opened.

During the operative preparation each dog received by intravenous infusion an amount of water equivalent to 3 to 5 per cent of its body weight. The water was administered as one of 5 different concentrations of NaCl solution: 0.5, 0.85, 1.5, 3.0 or 5.0 per cent. After this infusion had been administered, suitable 'priming' doses of

<sup>4</sup>Ladd and Raisz (12) have pointed out quite clearly that dog and man cannot necessarily be compared with respect to the renal handling of sodium.

<sup>5</sup>In two experiments (25, 26) clamps were placed on both renal arteries but the one on the left remained open throughout the procedure.



TABLE 1. EFFECT OF CONSTRICTION OF RIGHT RENAL ARTERY ON FUNCTIONS OF RIGHT<sup>1</sup> KIDNEY WITH SIMULTANEOUS ESTIMATION OF THE SAME FUNCTIONS OF LEFT KIDNEY RECORDED FOR COMPARISON<sup>2</sup>

DOG NO.	NO. PERIODS AV.	$C_F$ L		$C_{PAH}$ L		$P_{Na}$	$V$ L		$U_{Na}$ L		$UV_{Na}$ L		$REAB$ L	
		R	L	R	L		R	L	R	L	R	L	R	L
		cc/min.		cc/min.		mEq/l.	cc/min.		mEq/l.		μEq/min.		mEq/min.	
I	3	41	44	134	149	157	1.32	1.40	204	208	269	288	5.9	6.3
	3	46	48	146	147	157	1.32	1.54	222	221	293	338	6.6	6.8
	3	44	48	139	151	157	2.18	2.42	218	219	475	530	6.1	6.6
3	3	49	45	131	130	159	3.11	2.33	191	178	594	414	6.8	6.4
	3	48	46	136	144	158	2.54	2.65	216	199	547	527	6.7	6.4
	3	48	47	143	138	158	2.29	2.18	219	206	534	450	6.7	6.6
28	3	36	35	78	75	153	0.62	0.43	204	224	128	95	5.1	5.0
	3	34	34	79	82	153	0.58	0.67	206	196	119	130	4.8	4.8
	2	35	36	78	80	153	0.94	0.79	172	172	163	136	4.9	5.1
14	3	54	54	169	162	141	0.73	0.65	165	161	119	106	7.1	7.1
	3	52	52	182	186	141	0.97	1.05	141	144	136	151	6.8	6.8
	3	51	52	176	179	141	1.42	1.36	113	116	159	156	6.7	6.8
10	2	38	37	83	84	136	0.27	0.21	71	26	19	5	5.0	4.9
	3	37	39	83	82	138	0.22	0.21	36	36	8	8	4.8	5.1
	2	37	35	83	81	137	0.29	0.22	61	37	17	8	4.8	4.5
15	3	50	51	141	136	161	4.24	4.31	196	193	829	832	6.8	7.0
	3	40	45	120	135	161	2.77	4.66	153	144	427	670	5.7	6.2
	3	39	43	138	139	164	3.65	4.71	135	135	492	636	5.6	6.1
11	3	35	37	111	109	162	1.98	1.67	271	287	529	474	4.9	5.2
	1	25	42	87	133	164	0.98	2.87	239	232	234	666	3.7	5.9
	3	35	34	115	123	165	1.70	3.98	219	190	369	749	5.1	4.6
	3	33	34	124	122	169	3.03	4.27	172	171	521	732	4.8	4.7
21	2	37	37	84	81	164	0.79	1.04	212	216	166	225	5.6	5.5
	2	34	17	80	34	165	0.95	0.42	215	211	203	89	5.1	2.6
	2	38	30	85	84	166	0.91	0.99	217	216	198	213	5.8	4.5
12	2	63	68	208	196	161	5.07	5.74	207	206	1052	1185	8.6	9.2
	2	38	56	171	180	163	0.53	5.14	63	166	33	853	5.8	7.8
	2	51	56	154	171	164	1.95	5.01	146	163	284	815	7.7	7.9
26	3	54	53	148	152	179	4.40	5.34	204	176	894	938	8.3	8.1
	2	17	58	44	132	181	0.35	5.19	169	195	58	1010	2.9	9.0
	2	44	53	118	116	182	3.25	5.31	151	188	490	995	7.1	8.2

<sup>1</sup>In experiment 2 the clamp was placed about the left renal artery instead of the right.

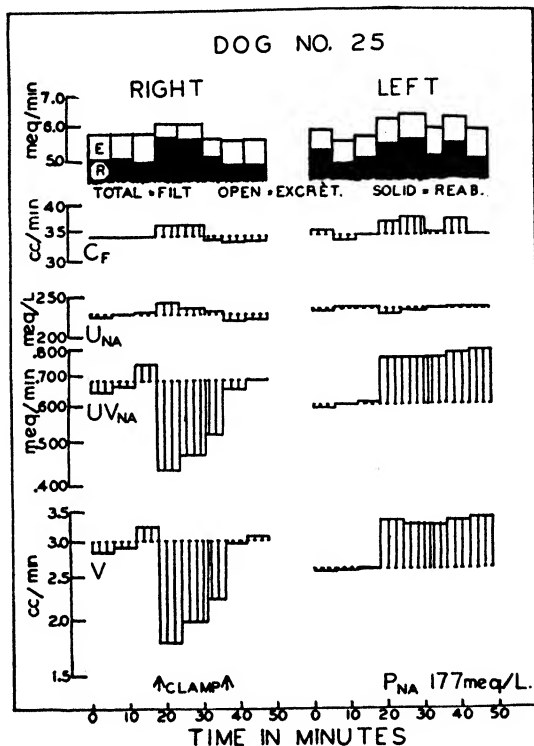
<sup>2</sup>The functions reported include glomerular filtration rate,  $C_F$ ; clearance of PAH,  $C_{PAH}$ ; sodium concentration in plasma,  $P_{Na}$ ; urine flow,  $V$ ; sodium concentration in urine  $U_{Na}$ ; rate of sodium excretion,  $UV_{Na}$ ; and rate of sodium reabsorption,  $REAB$ .<sup>3</sup> Column for right kidney is headed R, for the left, L. Placing of figures in italics indicates that these values were obtained during constriction of renal artery.

<sup>3</sup>Rate of sodium filtration was approximated by the calculation  $C_F \times P_{Na} \times 0.95$ .

creatinine, sodium *p*-amino-hippurate (PAH), and, when specified, glucose and diodrast, were given, followed by a 'sustaining' infusion to maintain stable plasma concentrations of these substances. The sustaining infusion was started at least 20 minutes prior to the collection of urine samples. The NaCl concentration was the same as that of the initial infusion and the rate of administration was approximately 3.0 cc/minute. The rate of infusion was 6.0 cc/minute in the experiments in which the tubular transfer maximum of glucose ( $Tm_g$ ) was estimated. The clearance techniques

Fig. 1. COMPARISON OF PERIOD-TO-PERIOD CHANGES between right (test) and left (control) kidneys in dog 25. With respect to top-most bar graph, total height of column (if completed to zero line) represents sodium filtered in mEq/min.; height of blank segment (*E*), rate of sodium excretion; and height of shaded portion (*R*), rate of sodium reabsorption.

Also indicated are glomerular filtration rate ( $C_F$ ), urine flow (*V*), urine sodium concentration ( $U_{Na}$ ) and rate of sodium excretion ( $UV_{Na}$ ). Arrows ( $\uparrow\uparrow$ ) indicate time of constriction and release, respectively, of right renal artery.



used and analytical procedures for estimating creatinine, PAH, glucose, diodrast and sodium concentrations were identical to those previously described (13).

## RESULTS

The data from 10 experiments are recorded in table 1 and from one experiment (25) in figure 1. Observations on filtration rate and the percentage of filtered sodium excreted in 4 of the experiments (1, 3, 28 and 14) listed in table 1 are presented in figure 2 in order to compare graphically the differences between right and left kidney function during the time of partial arterial occlusion. In these 5 experiments (25, 1, 3, 28 and 14) there was no change in glomerular filtration rate which could be attributed to tightening the clamp. In 2 (25 and 1) there was a slight increase in filtra-

tion rate and in 3 (3, 28 and 14) the decrease was less than 5 per cent. (See also table 1.) With the exception of *experiment 25* the changes in sodium and water excretion are small and somewhat obscured by either a progressive increase (1 and 14) or decrease (3) in sodium and water excretion during the experiment. However, if the changes in these functions during clamping are compared to the changes occurring simultaneously in the control kidney, it will be seen (fig. 2) that there is invariably a relative decrease in the excretion of sodium and water from the test kidney. This would not be expected on the basis of random variation. In *experiment 25* (fig. 1)

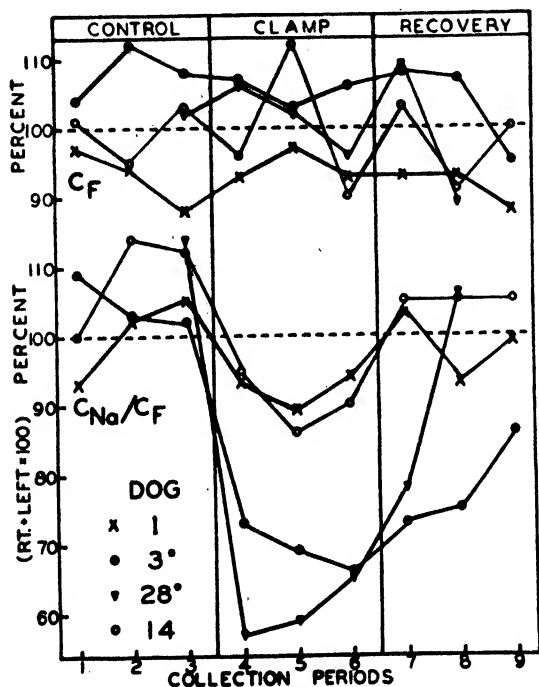


Fig. 2. FILTRATION RATE ( $C_F$ ) and percentage of filtered sodium excreted ( $C_{Na}/C_F$ ) in right kidney expressed as percentages of the same functions estimated simultaneously in left kidney (\*). In these 2 experiments percentage of filtered sodium excreted in right kidney was about 140 per cent of that in left during control periods. In order to approximate 100 per cent control base line 30 units were subtracted from each value throughout in each experiment. Corrections otherwise unnecessary.

the decrease in the excretion of sodium during partial arterial constriction is obvious and is sufficient to demonstrate an increased rate of sodium reabsorption.

In two experiments (10 and 15, table 1) filtration rate was moderately reduced during the time of arterial constriction. It cannot be stated whether or not this was a direct result of the clamping. Urine flow and sodium excretion fell off during the periods of partial occlusion in both and rose following release of the clamp without any increase in filtration rate. In another experiment (11, table 1) there was a definite drop in filtration rate during the first urine collection period of clamping but in the subsequent three periods of clamping the filtration rate returned to control values. Despite this sodium excretion remained low and returned to control values only when the clamp was released.

In the 8 experiments mentioned so far (25, 1, 3, 28, 14, 10, 15 and 11) the con-

centration of sodium in the plasma was normal<sup>6</sup> in 2 (14 and 10) and elevated in the other 6. In the 2 experiments in which the sodium concentration was normal there was a drop in the urine concentration of sodium during clamping when compared to the urine concentration of sodium in the opposite kidney. In *experiment 14* this change was of questionable significance. In the 6 experiments in which the concentration of sodium in the plasma was elevated arterial constriction invariably brought about an increase in the urine concentration of sodium when compared to the opposite kidney. In 2 experiments (1 and 3) the changes were within the limit of error of the method, nonetheless followed the pattern of the other 4 experiments. This occurred regardless of whether or not filtration rate decreased at the same time. In other words, the response of the sodium concentration in the urine to clamping appeared to depend on the plasma concentration of sodium rather than on whether or not there was a small change in the filtration rate.

In the other 3 experiments recorded in table 1 (12, 2 and 26) the filtration rate was markedly reduced by arterial constriction. In all 3 there was a fall in urine sodium concentration during clamping despite high sodium levels in the plasma. In all the percentage of filtered sodium reabsorbed was increased but the rate in mEq/minute was reduced as a result of the diminished amount of sodium filtered. It is obvious from table 1 that when filtration rate is reduced by 30 per cent or more, there is ordinarily a greater percentile drop in the excretion of water and salt than in filtration rate. It is also apparent that the filtration rate responds to the release of the clamp more rapidly than the excretion of water and salt.

In order to evaluate these data with respect to the so-called glomerulo-tubular imbalance for glucose 4 tests were conducted to obtain the filtration rate in relation to  $Tm_G$  (table 2), the transfer maximum of glucose. In *experiment 17* the reduction in urine flow and sodium excretion occurred without any measurable decrease in the  $C_r/Tm_G$  ratio, the measure of balance between glomerular filtration and normally functioning tubules. When the ratio was reduced, as is *experiment 21*, the effect on urine flow and sodium excretion was marked.

#### DISCUSSION

The present studies confirm the hypothesis that alterations in sodium reabsorption and excretion may be brought about by intrarenal mechanisms other than change in filtration rate. In 5 experiments there was no demonstrable reduction in filtration rate with partial arterial occlusion but there was a consistent reduction in sodium excretion when the changes were related to simultaneous estimates in the control kidney. In 4 of these experiments the changes are small but cannot be related to random variation with the exception of *experiment 1*. There is no doubt that undemonstrable changes in filtration rate could account for the decreased sodium excretion if a relatively constant rate of reabsorption is assumed. Further, it is possible that tightening the clamp on the right renal artery augmented sodium excretion from the left kidney, thereby invalidating any comparison between the two kidneys. However, these objections to the conclusions drawn cannot be invoked in *experiment 25* in which the changes observed are significant.

<sup>6</sup> Actually somewhat below normal for the dog.

In the data obtained there is evidence of the independent role played by the concentration of sodium in the plasma and/or tubular urine. In the 8 experiments in which filtration rate was changed little or not at all by arterial constriction, the change in the urine concentration of sodium appeared to depend on the plasma concentration of sodium rather than on any change in filtration rate. With respect to the second variable, viz. the rate of urine flow through the tubules little can be said which would indicate independence from glomerular filtration rate.

The reduction in sodium excretion without change in filtration rate which occurred during minimal arterial constriction was associated with a decreased rate of flow through the tubular lumen. However, no causal relationship has been demon-

TABLE 2. EFFECT OF CONSTRICTION OF RIGHT RENAL ARTERY ON FUNCTIONS OF BOTH KIDNEYS, INCLUDING GLUCOSE TRANSFER MAXIMUM,  $Tm_G$ ; AND DIODRAST TRANSFER MAXIMUM,  $Tm_D$ , (Experiment 17 only)<sup>1</sup>

DOG NO.	NO. PERIODS AV.	$C_F$		$Tm_G$		$C_F/Tm_G$		$V$		$U_{Na}$		$UV_{Na}$		$Tm_D$	
		R	L	R	L	R	L	R	L	R	L	R	L	R	L
		cc/min.		mg/min.				cc/min.		mEq/l.		$\mu$ Eq/min.		mg/min.	
17	3	44	40	146	135	.30	.30	6.03	4.44	92	87	554	390	11.1	11.0
	2	38	37	123	125	.31	.30	3.21	3.77	63	72	204	270	11.4	11.0
	2	38	34	136	125	.28	.27	4.38	3.57	68	60	297	215	11.0	11.0
														$P_{Na}$ mEq/l.	
20	3	27	27	57	54	.47	.50	3.92	4.37	29	36	114	159	128	
	3	20	27	51	50	.39	.54	2.54	5.46	7	44	17	238	130	
	2	22	25	50	44	.44	.57	3.21	4.62	19	38	60	176	130	
21	3	57	61	89	96	.64	.64	4.21	5.56	61	80	255	445	141	
	3	36	58	107	99	.34	.59	1.46	5.51	2	78	3	431	143	
	2	46	53	92	96	.50	.56	2.73	4.65	35	73	94	340	145	
22	3	29	30	59	59	.49	.51	5.66	6.11	69	70	391	430	135	
	2	8.4	28	33	49	.25	.57	0.76	5.74	2	62	2	354	136	
	2	17	24	37	38	.46	.63	3.40	5.29	35	50	120	265	137	

<sup>1</sup>For explanation of other symbols see table 1 and text.

strated. The results of Gesell's studies (14) suggested that decreased pulse pressure might be a factor in producing these changes but recent work by Selkurt (15) does not support such a conclusion.

There is no doubt that when the rate of tubular flow is reduced as a result of decreased filtration rate with a reduction in  $C_F/Tm_G$  ratio (table 2) there is a marked reduction in Na excretion and an increase in the percentage of filtered sodium reabsorbed. This effect has been well demonstrated by others (16-18).

#### SUMMARY

Fifteen experiments have been conducted on as many dogs for the purpose of assessing the role played by the filtration rate and other variables in the renal handling of sodium and water. The animals were anesthetized, the ureters exterior-

ized with plastic tubing, and a Goldblatt clamp was placed about the right renal artery through an incision in the right flank. The animals received hypotonic, isotonic or hypertonic saline solution by vein throughout the preparatory period and during the experimental procedure. The usual clearance techniques were employed to estimate renal function. The left kidney served as a control throughout the test, and control urine-collection periods with the clamp open were obtained both before and after constriction of the right renal artery. Minimal constriction of the renal artery with no measurable decrease in filtration rate was attended by decreased urine flow and sodium excretion, sufficient in *experiments 11* and *25* to demonstrate increased rate of sodium reabsorption. If only intrarenal mechanisms are to be considered, then one must postulate for these experiments some factor or factors other than alteration of filtration rate for the regulation of sodium reabsorption and excretion. The concentration of sodium in the plasma and the rate of flow of urine through the tubule have been suggested as such factors.

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# STUDIES ON RENAL PRESSOR MECHANISM AND ON VASCULAR REACTIVITY DURING DEPRESSOR RESPONSE TO ACUTE INFLAMMATION IN DOGS

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THE induction of an acute inflammatory reaction in hypertensive dogs frequently results in a decrease of the blood pressure to normal levels for several days, and sometimes for several weeks (1-3). The mechanism of this depressor response is at present unknown. Elucidation of the factors involved in this fall in blood pressure should be of value in understanding the hypertensive process. Further, it might aid in the development of techniques for maintaining the blood pressure at normal levels for longer periods of time.

Previous work from this laboratory has shown that during the depressor response to acute inflammation in hypertensive dogs the cardiac output tends to remain at control levels (4). The depressor effect of acute inflammation must therefore be attributed to a decrease in the peripheral resistance. The mechanism of this reduction in total peripheral resistance has not been clarified. Among several possibilities (2), the depressor response may be due to changes in the reactivity of the blood vessels during the acute inflammatory reaction, or to changes in the renal pressor mechanism. In order to evaluate these two possible mechanisms, the blood pressure responses to renal pressor agents and several other vasoactive drugs were tested in normal and in hypertensive dogs before and after the induction of an inflammatory reaction.

## METHODS AND MATERIALS

Two groups of dogs, 8 normotensives and 8 hypertensives, were used. The blood pressure responses to the renal pressor agents and other vasoactive drugs were measured in both groups before the induction of the inflammatory reaction. After adequate control studies, 2.5 cc. of turpentine were injected subcutaneously. This resulted in every instance in the production of local inflammation, going on to a fluctuating abscess within two or three days. During this period the blood pressure usually fell to normal levels (1, 2). Vascular reactivity was retested one to three days following the injection of turpentine in all animals. In 4 of the 8 hypertensive animals the vascular reactivity was evaluated with the same vasoactive agents

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daily for three consecutive days, beginning on the second day after the injection of turpentine.

Of the 8 hypertensive dogs 6 had had bilateral constriction of the renal arteries with linen ligatures (5). Two were spontaneously hypertensive according to the criteria previously described (4). All hypertensive dogs were trained to lie quietly on the animal board. Blood pressure was taken by puncture of the femoral artery and recorded on a photokymograph with the Hamilton manometer (6). After a control blood pressure was taken, 5 units (U) of angiotonin (hypertensin)<sup>3</sup> were given intravenously (i.v.), while continuous pressure and pulse rate tracings were recorded. Five to 10 minutes later, when the blood pressure had returned to control levels, renin<sup>4</sup> (2 U) was injected i.v. and another continuous record taken.

In the normotensive dogs, the tests were done under general anesthesia induced by i.v. sodium pentobarbital (25 mg/kg.). The depth of anesthesia was maintained by repeated small injections of sodium pentobarbital solution (25 mg/cc.) throughout the experiment. Thus the blood pressure was maintained at a relatively constant level. The femoral artery was cannulated and connected with a mercury manometer to record mean arterial pressure. A constant slow i.v. infusion of 0.9 per cent saline solution (1 cc/min.) was given through a femoral vein. This was instituted to facilitate i.v. administration of the vasoactive drugs. After these preparations were completed, the control levels of arterial pressure and pulse rate were recorded. The vasoactive drugs, epinephrine (1/40,000 mg.), pitressin (0.5 U), tetraethylammonium chloride (TEA, 2.5 mg/kg.), angiotonin (5 U) and renin (2 U) were given in the order listed. After each drug, the mean arterial pressure and pulse rate were recorded every  $\frac{1}{2}$  to 1 minute until both had returned to control levels. The renin was tested last because of its prolonged pressor effect.

## RESULTS

*Blood Pressure Responses in Unanesthetized Hypertensive Animals (Table 1).* Among the 8 hypertensive dogs, 7 showed a definite fall of blood pressure after the induction of turpentine abscess (table 1). In these the systolic pressure reduction ranged from 20 to 100 mm. Hg, averaging 32. The reduction in diastolic pressure ranged from 15 to 60 mm. Hg, with an average of 26. These depressor responses are statistically significant (table 2).

During both the control period and the acute inflammatory phase, the injection of 5 U of angiotonin caused a rise of both systolic and diastolic pressures of 10 to 50 mm. Hg within 20 to 40 seconds (table 1). Over the course of the next 2 to 3 minutes the blood pressure gradually returned to the control level. The average rise of pressure in response to the 5 U of angiotonin in the control period was 21/26 mm. Hg, and in the acute inflammatory phase was almost identical, 20/26 mm. Hg (table 2). The average time to the peak rise in the control experiments was 28 seconds and

<sup>3</sup> We wish to express our appreciation to Dr. O. Helmer of Eli Lilly & Co., Indianapolis, Ind., who kindly supplied the angiotonin used in these studies.

<sup>4</sup> This was obtained through the kindness of Dr. G. E. Wakerlin of the Department of Physiology, University of Illinois School of Medicine, Chicago, Ill.



the duration of the pressor response was 140 seconds. During acute inflammation these time measurements were 32 and 142 seconds, respectively.

Similarly the injection of 2 U of renin elicited an essentially equal pressor response in the control period and after the induction of abscess (table 1). It caused an average rise of blood pressure of 19/28 mm. Hg in the control, and 26/29 mm. Hg during the inflammatory phase (table 2). The average time for the pressure to reach the maximum in the control period and during acute inflammation was 76 and 86 seconds, respectively.

TABLE 1. PRESSOR RESPONSE OF UNANESTHETIZED HYPERTENSIVE DOGS TO ANGIOTONIN AND RENIN BEFORE (CONTROL) AND DURING (EXPERIMENTAL) ACUTE INFLAMMATORY REACTION

	CONTROL					EXPERIMENTAL			
	Blood Pressure (mm.Hg)					Blood Pressure (mm.Hg)			
	Dog No.	Systolic	$\Delta$ systolic	Dias-tolic	$\Delta$ dias-tolic	Systolic	$\Delta$ systolic	Dias-tolic	$\Delta$ dias-tolic
Angiotonin <sup>1</sup>	Z19	220	+30	105	+45	175	+45	75	+40
	Z20	215	5	120	15	220	15	100	35
	Z14	225	40	115	45	205	30	90	45
	Z77	225	50	140	40	220 <sup>2</sup>	55	130 <sup>2</sup>	30
	Z80	260	10	130	15	165	20	90	20
	Z13	250	10	120	25	190	30	90	35
	Z94	260	10	125	10	185	5	90	15
	Z40	250	10	125	10	155	5	65	10
Renin <sup>1</sup>	Z19	225	10	125	25	190	10	100	20
	Z20	200	0	120	5	210	15	110	20
	Z14	225	50	130	40	200	25	90	40
	Z77	250	30	140	50	220	50	125	35
	Z80	265	20	140	25	185	35	90	35
	Z13	250	10	120	40	200	50	95	45
	Z94	260	20	125	15	210	25	110	25
	Z40	275	15	120	25	180	30	80	15

<sup>1</sup> Fifteen experiments with angiotonin and 12 with renin were done on these 8 dogs; a representative experiment with each dog is cited; other experiments yielded similar results (see text); experiments with renin were done after those with angiotonin, adequate time being allowed for the blood pressure to stabilize at or near pre-injection levels following the angiotonin-induced pressor response.

<sup>2</sup> Poor depressor response to acute inflammatory reaction.

*Blood Pressure Responses in Anesthetized Normotensive Dogs (Table 3).* In 2 of the 8 normotensive dogs the mean arterial pressures were measured before and after the induction of inflammation. Both showed a lowering of blood pressure of 14 to 30 mm. Hg after the abscess developed. The remaining experiments in this part of the study were carried out on animals without measurements of the pre-inflammatory blood pressure. The pressor responses to i.v. injection of epinephrine (1/40,000 mg.), pitressin (0.5 U), angiotonin (5 U), and renin (2 U) were not significantly different before and after the induction of abscess (table 3).

Tetraethylammonium chloride (2.5 mg/kg.) intravenously elicited a fall in blood pressure of 42 mm. Hg in the pre-inflammatory period. After the induction

of the inflammatory reaction an average fall of 52 mm. Hg was seen when the TEA was given (table 3). This difference between control and experimental depressor responses to TEA is not statistically significant. Thus an essentially similar TEA induced fall in pressure occurred even though the initial blood pressure in the post-inflammation group was lower than in the pre-inflammation group.

## DISCUSSION

A fall of arterial blood pressure can be attributed to a decrease of either or both of two factors: the cardiac output and/or the total peripheral resistance. During acute pyrogenic reactions in normal and hypertensive human subjects Grollman (7) and Bradley *et al.* (8) found that the cardiac output is increased. Stamler, Fishman, Katz and Rodbard (4), working with unanesthetized hypertensive dogs, found that the cardiac output did not change significantly during the sustained depressor

TABLE 2. SUMMARY OF PRESSOR RESPONSES OF UNANESTHETIZED HYPERTENSIVE DOGS TO ANGIOTONIN AND RENIN BEFORE AND DURING ACUTE INFLAMMATORY REACTION

	CONTROL				EXPERIMENTAL			
	Blood Pressure (mm.Hg)				Blood Pressure (mm.Hg)			
	Systolic	$\Delta$ systolic	Diastolic	$\Delta$ diastolic	Systolic	$\Delta$ systolic	Diastolic	$\Delta$ diastolic
Angiotonin <sup>1</sup>	238 $\pm$ 5.0 <sup>2</sup>	21 $\pm$ 6.0	123 $\pm$ 2.6	26 $\pm$ 5.1	206 $\pm$ 7.5	20 $\pm$ 3.5	97 $\pm$ 4.3	26 $\pm$ 2.9
Renin <sup>3</sup>	243 $\pm$ 9.3	19 $\pm$ 5.4	124 $\pm$ 4.6	28 $\pm$ 5.2	209 $\pm$ 6.2	26 $\pm$ 4.2	100 $\pm$ 4.5	29 $\pm$ 2.7

<sup>1</sup> Eight control and 15 experimental tests.      <sup>2</sup> The standard error (S.E.) is 5.0, calculated from

the formula,  $S.E. = \frac{S.D.}{\sqrt{N}}$  where N is the number of tests and S.D. is the standard deviation,  

$$S.D. = \sqrt{\frac{\sum(\bar{X} - X)^2}{N - 1}}$$

<sup>3</sup> Eight control and 12 experimental tests.

response to an inflammatory process. Therefore under these experimental conditions there is no evidence that the depressor effect of acute inflammation is due to a decrease of cardiac output. Hence the blood pressure fall must be due to a lowering of the total peripheral resistance.

The mechanism by which the peripheral resistance is reduced during an inflammatory reaction is not known. Several possible mechanisms, operating via either humoral or nervous routes, may be examined in relation to the findings in the present study. Among humoral mechanisms, the renal pressor system must first be considered. The fall of blood pressure induced by abscess formation may conceivably be due to a reduction of renin titre, to a reduction of plasma pre-angiotonin (hypertensinogen) content, or to a lowered vascular reactivity to circulating angiotonin.

Renal ischemia is believed to be associated with increased production of renal pressor material. The induction of an acute abscess is almost always associated with an increase in renal blood flow (2, 9). However, no necessary temporal relationship has been demonstrated between the renal hyperemia and the fall in blood pressure (2). It is unlikely, therefore, that the depressor response is due to a decrease of renin production secondary to relief of renal ischemia.

Nor do our results substantiate the possibility of a decreased hypertensinogen titre during the acute inflammatory process. In all our experiments the response to renin was the same before and after the injection of the turpentine. If there had been a reduction in plasma hypertensinogen level, the response to renin should have been diminished after the induction of abscess.

Further, the results reveal that there was no significant difference between the blood pressure response to hypertensin before and during acute inflammation. This militates against the possibility of a lowered reactivity of the blood vessels to the renal pressor principle during the acute inflammatory phase.

TABLE 3. BLOOD PRESSURE RESPONSE TO VASOACTIVE AGENTS BEFORE AND DURING ACUTE INFLAMMATORY REACTION IN ANESTHETIZED NORMOTENSIVE DOGS

AGENT	DOSE	INFLAMMATORY REACTION	NO. OF DOGS	NO. OF EXPER.	AV. MEAN ARTERIAL PRESSURE PRE-TEST	BLOOD PRESSURE RESPONSE	DURATION OF RESPONSE
					mm. Hg	mm. Hg	min.
Epinephrine	1/40,000 mg.	Before	6	7	123 ± 12.4 <sup>1</sup>	36 ± 5.7	2.0
		During	4	4	114 ± 10.6	52 ± 12.4	2.0
Pitressin	0.5 U	Before	4	4	146 ± 17.1	10 ± 2.3	3.7
		During	4	4	111 ± 10.1	26 ± 7.1	6.5
Angiotonin	10 U	Before	5	5	127 ± 15.6	26 ± 4.1	3.8
		During	4	4	116 ± 11.2	33 ± 7.6	4.0
Renin	2 U	Before	3	5	143 ± 10.4	13 ± 2.8	13.0
		During	4	5	111 ± 8.6	20 ± 4.8	over 10.0
TEA <sup>2</sup>	2.5 mg/kg.	Before	4	4	138 ± 13.3	-42 <sup>3</sup> ± 12.5	5.1
		During	4	4	115 ± 9.4	-52 <sup>3</sup> ± 6.2	6.5

<sup>1</sup> The standard error is 12.4. <sup>2</sup> TEA = tetraethylammonium chloride. <sup>3</sup> - = a drop in pressure; all others indicate a rise.

Among other humoral factors, the role of adrenal cortical hormones must be evaluated. Williams *et al.* (10) and Friedman *et al.* (11) showed that in adrenalectomized rats and dogs respectively there is a diminution in response to renin. Remington and his associates (12) further demonstrated that withdrawal of sustaining cortical extract in adrenalectomized dogs was followed by a partial loss of sensitivity to renin, pitressin, barium chloride and small doses of epinephrine. Such a diminution of vascular sensitivity to renin, pitressin and epinephrine was not found in our experiments during an acute inflammatory reaction. Hence our data do not support an hypothesis that the hypotensive effect of abscess induction is due to a diminution of available adrenal cortical hormones. This conclusion is in accord with other findings during the depressor response to acute inflammation (13).

Among the nervous factors, a general lowering of sympathetic tone during the acute inflammation may also conceivably cause a general vasodilation, and a reduction of total peripheral resistance. Our results with TEA, however, do not support

this hypothesis. This sympatholytic (as well as parasympatholytic) drug caused a quantitatively similar fall of blood pressure in dogs before and after abscess induction. Had there been a reduced sympathetic tone, the blood pressure should have been affected to a lesser degree in the dogs having abscesses.

We have limited ourselves to a consideration of the most obvious mechanisms that may effect a fall in peripheral resistance. Our experimental results suggest that the fall in blood pressure during acute inflammation is due to none of these factors. It is of course possible that our methods for testing the responsiveness of the blood vessels are inadequate. On the other hand the possibility remains that the depressor effect of acute inflammation is due to factors that induce a general reduction of peripheral resistance without changing the vascular reactivity to pressor agents.

#### SUMMARY

The depressor response to acute inflammation was analyzed in order to determine if the fall in blood pressure was due to a change in the reactivity of the blood vessels. Possible effects upon the renal pressor mechanism were also investigated. Hypertensive and normotensive dogs were used. The response to renin and angiotonin was identical in both the pre- and post-inflammatory periods. The responses to other pressor agents (epinephrine and pitressin) and to tetraethylammonium chloride were unchanged by the induction of an acute inflammatory reaction. These results indicate that the observed reduction in total peripheral resistance is not due to changes in the reactivity of the blood vessels to these vasoactive principles. The implications of these results are discussed.

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# REABSORPTION OF BROMIDE BY THE KIDNEY

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WHILE it is the opinion of the majority of authors (1-5) that the kidney does distinguish between chloride and bromide ions, others disagree (6, 7). Their conclusions were based on comparisons of the composition of blood and urine, and no one, apparently, has computed the amounts of bromide actually filtered and reabsorbed. The following experiments were undertaken to enlarge our knowledge in this respect.

## METHODS

Two groups of 4 experiments each were carried out on 5 dogs, all healthy females, weighing from 7 to 16 kg. In the first group of experiments the dogs were starved but allowed free access to water for 48 hours; the day before the experiment, they were given, by stomach tube, sodium bromide, 10 mm/kg. of body weight dissolved in a liter of water. This ensured maximum tubular reabsorption of halide. Food was not withheld in the second group, but on the day before the experiment, the dogs were given sodium bromide, 10 mm/kg. of body weight and, in addition, 10 gm. of sodium chloride. In one instance this amount of sodium chloride was given 48 hours before the experiment as well as on the day before. In these animals reabsorption of halide by the kidney tubules was less complete. Forty-five minutes before beginning an experiment each animal received 75 ml. of water/kg. by stomach tube, and following this, 30 mg/kg. of sodium pentothal intraperitoneally. Urine was collected by a catheter inserted into the bladder. Filtration rate was measured by a modification of the method of Smith (cf. 8), using creatinine instead of inulin. The priming dose of creatinine used was 0.5 gm. dissolved in 25 ml. of saline; following this a 1.4 per cent solution was infused at a rate of 1.5 ml/minute. Blood samples, collected under mineral oil were analyzed for creatinine by a photoelectric modification of the method of Folin and Wu. Total serum halides were measured by the method of Sordroy (9) after precipitation of the proteins with zinc hydroxide.

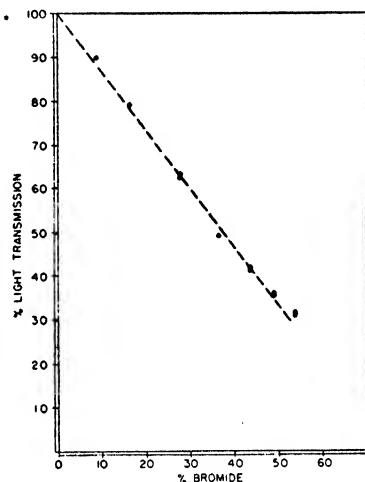
Both Walter's (10) gold chloride method for bromides and the popular modification of it by Wuth (11) have been found to be very unsatisfactory (12, 13). This is partly because the color is affected by the presence of chlorides (13), and partly because the brownish yellow of gold bromide, in itself difficult to read, is to a considerable extent obscured by an excess of gold chloride. Other anions such as tungstate were found to affect the color, as did also the pH. Moreover, the color does not follow Beer's law. Fortunately, however, it was found that if the total halide concentration

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is kept constant, the percentage light transmission (not optical density), measured photoelectrically, is proportional, within wide limits, to the percentage of total halide present as bromide.<sup>1</sup> The following simple method based on these findings proved to yield satisfactory results.

Filtrate from the zinc hydroxide precipitation is diluted until it contains 5 mEq. total halide/l. (If the original serum contained  $x$  mM total halide/l., 2 ml. of filtrate should be made up to  $x/50$  ml. with distilled water. Similarly 2 ml. urine is diluted to  $x/500$  ml.). To 2 ml. of this diluted filtrate or urine in colorimeter tubes are added 5 ml. water, 2 ml. of a 0.0375 per cent gold chloride solution, and one ml. of 20 per cent acetic acid, and readings are made as soon as convenient. The reagent blank contains 2 ml. of 5 mM sodium chloride instead of filtrate. When necessary,

Fig. 1. RELATIONSHIP OF BROMIDE concentration as percentage of total halide to percentage light transmission. Points re from recovery experiments in which varying amounts of bromide were added to blood serum before precipitation.



standards may be made from 20 mM solutions of sodium chloride (1.169 gm/l.) and sodium bromide (2.059 gm/l.), mixed so as to give, e.g. 20 and 40 per cent of bromide in terms of total halide, and diluted to 5 mM/l. The percentage light transmittance ( $Y$ ) plotted against the percentage of bromide ( $X$ ) in the standards and reagent blank should yield a straight line, and results may be read directly from this. Using the Evelyn colorimeter we obtained a line fitted by the equation

$$X = 70.6 - 0.706Y$$

and results may be calculated from such a formula. The slope of the line, 0.706, was found to be directly proportional to the total halide concentration; it seems simpler, however, to dilute to a standard halide concentration than to correct by calculation. Recovery experiments in which bromide was added to serum before protein precipi-

<sup>1</sup> This probably indicates that the reaction is not a simple one, but it has not been sufficiently studied. Chemically it appears possible that not only may  $\text{AuBr}_2$  be formed, but also  $\text{AuBrCl}_2$  and  $\text{AuBr}_3\text{Cl}$ . Comparison of the absorption maxima with those of gaseous gold chloride indicates that the color-producing substances are probably of this type, not complex cations.

tation were satisfactory for all amounts of bromide up to 50 per cent of total halide, and showed no tendency to systematic deviation (see fig. 1).

### RESULTS

The filtration rate in the first series of experiments was  $55.3 \pm 18.3$  ml/minute<sup>2</sup>; in the second series it was  $50.1 \pm 8.9$  ml/minute. If the weights of the animals are considered, the rates were  $6.4 \pm 1.6$  and  $4.0 \pm 1.1$  ml/min/kg. respectively, a highly significant difference, probably to be attributed to the dehydration induced by the administration of large amounts of salt in the second series (cf. 14). The total halide

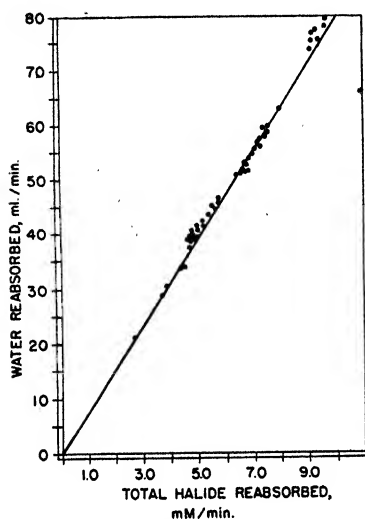


Fig. 2. RELATIONSHIP BETWEEN amounts of total halide and of water reabsorbed.

concentration of the plasma in the first series was  $124.8 \pm 3.0$  mM/l.; in the second series it was slightly but significantly higher, viz.  $127.6 \pm 3.4$  mM/l. In the low chloride series the bromide ion made up  $24.0 \pm 6.1$  per cent of the total halide; in the high chloride series it was  $13.8 \pm 1.6$  per cent. Differences were also seen in reabsorption; in the low chloride experiments  $99.5 \pm 0.20$  per cent of the halide filtered was reabsorbed; in the high chloride experiments this percentage fell to  $96.6 \pm 0.53$ .

A close relationship is known to exist between the rate of reabsorption of chloride and that of water, or what is, at least numerically, almost the same, the rate of filtration, the apparent reason being that the reabsorption of chloride is related to, and very probably dependent on, that of sodium which in turn is related to the reabsorption of water (15). On this basis, and since electrically and osmotically an ion of bromide is equivalent to one of chloride, it would be expected that the

rate of water reabsorption would be related, not to the reabsorption of either bromide or chloride separately, but to that of total halide. This was found to be the case (fig. 2). With each millimole of halide  $7.92 \pm 0.34$  ml. of water was reabsorbed.

The second problem is the relationship of bromide to chloride. On plotting the ratio bromide:chloride reabsorbed against the ratio of the amounts filtered (both in mM/min.) a linear relationship is evident (fig. 3). Points from the same experiment tend to fall very close together, often overlapping; and since the differences between them are only slightly greater than the experimental error, it is difficult to see such a relationship within individual experiments, although it is quite clear when all experiments are taken together. The equation of the straight line in figure 3, obtained by the method of least squares, is

$$\frac{\text{Br}_r}{\text{Cl}_r} = 0.987 \frac{\text{Br}_f}{\text{Cl}_f} + 0.00619 \quad (1)$$

<sup>2</sup> The figure following the  $\pm$  sign is standard deviation throughout this paper.

the subscripts  $r$  and  $f$  indicating reabsorbed and filtered, respectively. The first conclusion to be drawn is that the ratio bromide:chloride reabsorbed is almost completely determined by the ratio filtered; this probably indicates that the primary factor governing the relative amounts reabsorbed is the composition of the fluid to which the tubule cells are exposed. In the second place it is apparent that although the relationship is linear, the two ratios are not equal. Experimentally, the bromide:chloride ratio in the filtrate was nearly always (46 times out of 51) less than the ratio of the amounts reabsorbed; in the remaining five instances they were equal; and in no case was the ratio in the filtrate greater. While the differences between the ratios were slight, the fact that they were always in the same direction renders it very unlikely that they are due to experimental error; and statistically they were found to be highly significant. This is implied by *equation 1* from which it follows that the

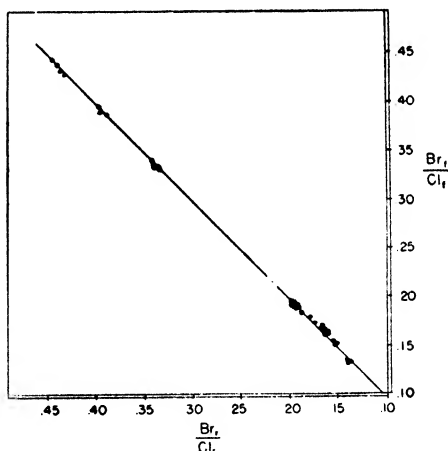


Fig. 3. RELATIONSHIP BETWEEN bromide:chloride ratios filtered and reabsorbed.

difference between the ratios is  $0.00619 - 0.013(Br_f/Cl_f)$ . This will be positive, i. e. the ratio reabsorbed will be greater for all values of  $Br_f/Cl_f$  less than 0.476 which is very rarely exceeded *in vivo*. Furthermore, the smaller  $Br_f/Cl_f$  the greater will the difference be; and since this ratio was nearly always smaller in the high chloride experiments, this conclusion is in agreement with the observation that the differences in the ratios were greater in the latter series of experiments.

Similar relationships were found between bromide and total halide.

*Equation 1* multiplied by  $Cl_r/Br_f$  becomes

$$\frac{Br_r}{Br_f} = 0.987 \frac{Cl_r}{Cl_f} + 0.00619 \frac{Cl_r}{Br_f} \quad (2)$$

It is obvious that this cannot hold for cases where either of the first two ratios would exceed unity. That it does hold very nearly, but not quite exactly, is shown by the accompanying table 1. According to this equation the fraction of bromide reabsorbed depends for the most part on the extent of chloride reabsorption, modified to a slight degree by a factor varying directly with the absolute rate of chloride reabsorption



and inversely with the amount of bromide in the filtrate. The biological significance of this appears to be that the mechanism which increases the rate of chloride reabsorption acts even more powerfully on the bromide ion. Moreover the smaller the percentage of bromide in the filtrate, the more nearly complete is its reabsorption. Both these conditions would be fulfilled if, as the filtrate passes down the tubule, the amount of chloride or bromide reabsorbed approached a maximum asymptotically (e.g. like a first or second order chemical reaction) and if the 'velocity constant' of the bromide reabsorption were somewhat greater than that of chloride.

The relationship of these findings to the problem of salt and water balance can be deduced by multiplying *equation 2* by the factor  $W_f/W_r$ ,  $W$  standing for water (ml/min.) filtered or reabsorbed as indicated by the subscript.

$$\frac{Br_r}{W_r} \cdot \frac{W_f}{Br_f} = 0.985 \frac{Cl_r}{W_r} \cdot \frac{W_f}{Cl_f} + 0.00619 \frac{Cl_r}{W_r} \cdot \frac{W_f}{Br_f} \quad (3)$$

The expression on the left is the ratio of bromide concentrations in the 'reabsorbate' and filtrate, ('bromide concentration in the reabsorbate' implying merely the relative

TABLE I.

EXPER.	Br <sub>r</sub> /Br <sub>f</sub> CALCULATED FROM EQUATION 2	FOUND	EXPER.	Br <sub>r</sub> /Br <sub>f</sub> CALCULATED FROM EQUATION 2	FOUND
1	0.996	0.997	5	0.974	0.979
2	0.996	0.997	6	0.992	0.987
3	1.000	0.998	7	0.997	0.985
4	0.998	0.998	8	0.984	0.985

quantities of these two substances reabsorbed). As the value of this term increases, a greater quantity of bromide, relative to water, is retained by the kidney; when its value falls more bromide, relative to water, is excreted. The first term on the right of the equation applies in the same way to chloride. By a process of reasoning similar to that given for *equation 2* it can be concluded—and is confirmed by experiment—that the bromide ratio usually exceeds and is never less than the chloride ratio. The conservation of bromide by the kidney, relative to the retention of water, is greater than is the case with chloride, and this difference is more marked, the greater the tendency to conserve halide, and less marked the greater the concentration of bromide in the plasma.

With regard to the problem whether the kidney is 'indifferent' to bromide ion, it appears that under conditions of nearly complete halide reabsorption, the kidney does not, at least in an overall sense, distinguish between the two ions, and in this respect von Wyss (6) and Frey (7) are confirmed. Apart from this, the kidney appears to reabsorb bromide more rapidly than chloride.

## SUMMARY

A simple and accurate modification of the gold chloride method for bromides is described. The total halide reabsorbed, rather than the chloride or bromide separately, is related to the water reabsorbed. The ratio bromide:chloride reabsorbed is

determined by the ratio bromide:chloride filtered and exceeds it except when there is practically complete reabsorption of both ions. An equation relating the two ratios is given. The percentage bromide reabsorbed is a function of the percentage chloride reabsorbed, the absolute amount of chloride reabsorbed, and the amount of bromide filtered. These findings can be explained on the assumption that bromide is reabsorbed somewhat more rapidly than chloride.

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# COMPARISON OF DIRECT WITH INDIRECT RENAL BLOOD FLOW, EXTRACTION OF INULIN AND DIODRAST, BEFORE AND DURING ACUTE RENAL NERVE STIMULATION<sup>1</sup>

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AMONG recent studies dealing with factors modifying the renal circulation Kottke, Kubicek and Visscher (1-3) have reported in a series of papers the effects of acute and chronic stimulation of the renal nerves. When measured indirectly by the clearance of creatinine and para-aminohippurate, glomerular filtration rate and renal blood flow (also measured with the thermostromuhr) were found to be reduced during renal nerve stimulation. Trueta and his collaborators (4, 5) and others (6-9) using injection methods have reported changes in the distribution of blood flow within the kidney during a variety of procedures, included among which was stimulation of the sciatic nerve. With the latter form of stimulation an effect on the kidney was inconstantly observed. Among the various reports an unpredictable and variable degree of cortical ischemia and juxtamedullary 'shunting' has been described.

The acute experiments reported here were performed in an attempt to determine the effects of direct renal nerve stimulation on renal blood flow, measured directly and continuously by a rotameter, on the clearance and extraction of inulin and diodrast, and on the distribution of blood flow within the kidney using a dye injection technique. In addition, this study afforded the opportunity to compare continuous direct flow measurements with those obtained by calculation from clearance and extraction data under control and experimentally altered conditions during stimulation and recovery.

## METHODS

Mongrel dogs, weighing from 10 to 16 kg., were anesthetized with pentobarbital sodium (30 mg/kg.) and suspended over the animal board in the prone position by hooks placed through the dorsal skin. In this position a better retroperitoneal exposure of the left renal pedicle could be made through the usual lumbar incision. The renal artery and the surrounding nerves were gently dissected free near the kidney and double loop electrodes were applied around these structures. To avoid undue trauma a search for all nerve filaments was not attempted. The ureter was cut and a double-lumen tygon catheter inserted so that the tip lay in the renal pelvis.

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<sup>1</sup> This material was presented at the Fall meeting of the American Physiological Society, Augusta, Georgia, September 14 to 17, 1949. Abstract, *Am. J. Physiol.* 159: 592, 1949.

Essentially all of the residual urine appeared to be removed from the renal pelvis by washing with 5 cc. of warm saline. The renal vein was isolated for subsequent cannulation and the spermatic or ovarian vein tied. Mean blood pressure was recorded from the right carotid artery by means of an optical manometer.

Heparin (5 mg/kg.) was given intravenously before carotid cannulation and blood pressure was observed during a priming infusion of 3 cc/kg. of a 5 per cent solution of pontamine fast pink.

The renal vein was cannulated directly without interruption of blood flow with a thin-walled aluminum cannula which was then snared in place with a string previously placed around the vein. The renal vein blood was passed through a large optical recording rotameter and returned via the left jugular vein. Before insertion of the cannula, renal vein pressure was  $0 \pm 0.5$  mm. Hg in the position in which the dog was suspended. The pressure drop across the rotameter, tubing and cannulae was 3.7 mm. Hg at 100 cc/minute and 8.1 mm. Hg at 200 cc/minute.<sup>2</sup>

Diodrast and inulin were given in priming and sustaining doses through a side tube interposed between the outflow orifice of the rotameter and the jugular cannula. Saline in amounts of approximately 100 cc/hour, pontamine fast pink, and, in some experiments, compatible dog plasma were also given through this tube.

Renal vein blood samples were drawn through a needle inserted directly into the rubber connection to the renal vein cannula. Arterial samples were drawn from a three-way stopcock attached to the carotid cannula. Urine collection periods for control, stimulation and recovery averaged 14 minutes, range 9.5 to 29.5 min. For hematocrit, diodrast and inulin extractions 5 cc. of arterial and renal venous blood were drawn from one to three times each period. Renal artery-nerve stimulation varied in different experiments from 4 to 9 volts sinusoidal alternating current at 2 to 11 cycles/second, and from 1 to 15 volts at 60 cycles/second. Diodrast and inulin were determined in urine, plasma and whole blood by modifications<sup>3</sup> of the methods of Corcoran and Page (13, 14).

Using a by-pass tube the rotameter was calibrated with the animal's own blood *in situ* at the beginning or in the middle and at the end of each experiment.

#### RESULTS

Twenty-two experiments were performed in 8 of which all of 9 variables were determined simultaneously. The trends of the results were the same in all experiments and they will be combined into one group for presentation.

As an example of a representative experiment, figure 1 is a reproduction of the original record showing renal blood flow and systemic arterial blood pressure changes during control, one period of stimulation and two recovery periods. The findings in this experiment are average for the group and they will be presented later in greater detail to illustrate the changes in individual variables.

<sup>2</sup> This degree of added resistance to venous outflow would not be expected to alter clearances or renal blood flow according to the work of Selkurt (10) and Blake (11). In addition, the average weight of the experimental kidney at the end of the experiment was less than 1% greater than that of the undisturbed control kidney indicating that no significant congestion had occurred (12).

<sup>3</sup> Plasmas were 'yeasted' prior to protein precipitation for inulin determinations. One per cent Duponol PC was used instead of 0.1% for whole blood diodrast determinations.

Stimulation of the renal nerves, with or without simultaneous stimulation of the renal artery,<sup>4</sup> invariably caused a decrease in renal blood flow. Stimulation caused no change in mean blood pressure in approximately one-half of the experiments, and in the remainder there was a transient increase within the first minute (av. 6 mm. Hg) which decreased to within 2 mm. of the control value after 2 minutes. Greater reductions in blood flow were obtainable by progressively increasing the stimulation in several stages from 2 volts to 10 volts AC. The decrease in blood flow was fairly well sustained, but often flow rose gradually to a slightly higher level. Figure 2 shows the effect of nerve (or artery-nerve) stimulation on the directly measured renal blood flow in 22 experiments with 28 periods of control. From control values averaging 118 cc/minute (2.8 cc/min./gm. of kidney; 8.8 cc/min./kg. body weight) renal blood flow diminished an average of 53 per cent during renal nerve stimulation and returned to an average of 86 per cent of control flow.

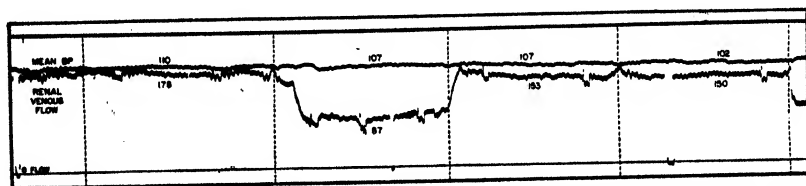


Fig. 1 REPRODUCTION OF A PORTION of an original record showing effects of renal nerve stimulation on renal blood flow and systemic BP. Vertical interrupted lines separate urine collection periods: a) control 14.5 min., b) stimulation 13.5 min., c) recovery I 13 min., d) recovery II 13 min. Stimulation was begun at 7.5 volts, 11 cps and increased 1.5 min. later to 10.5 volts, 11 cps. Order of curves from top: time—30 sec.; BP base line; mean carotid BP in mm. Hg; mean renal venous blood flow (rotameter) in cc/min.; zero flow base line. Transient decrease in blood flow record are artifacts caused by withdrawal of blood samples from renal vein cannula.

In 12 experiments (17 periods of stimulation) urine flow decreased simultaneously with blood flow, and in some instances essentially no urine was excreted (fig. 3). The more pronounced decreases in urine flow were associated with the greatest falls in directly measured blood flow.

In 18 periods, during which the renal nerves were stimulated at various intensities, the extraction percentage of diodrast from plasma diminished an average of 17 per cent below its control and returned during recovery periods to an average of 96 per cent of its control value (fig. 4). In many instances the drop in extraction percentage did not occur until late in the stimulation period, and occasionally the lowest extraction was observed during the early part of the subsequent recovery period. During 18 periods of control before stimulation the average of 29 plasma D extractions was 75 per cent. The extraction of inulin decreased an average of 38 per cent below its control value in 11 of 12 experiments, but in one it did not decrease (fig. 5). During recovery  $E_{OIN}^{\%}$  returned to an average of 99 per cent of the control value.

When comparing renal blood flows measured directly by the rotameter and

<sup>4</sup> As previously reported by Kottke (15), stimulation of the renal artery alone produced no demonstrable change in renal blood flow.

indirectly by chemical clearance methods, it was apparent that the latter were not adequate in several respects for acute experimental studies of the type reported here. To illustrate this and other findings, the experiment, part of which is shown in figure 1, has been summarized graphically in figure 6.

Fig. 2. GRAPHIC SUMMARY of direct renal blood flow changes (rotameter) during renal nerve stimulation and recovery (22 experiments).

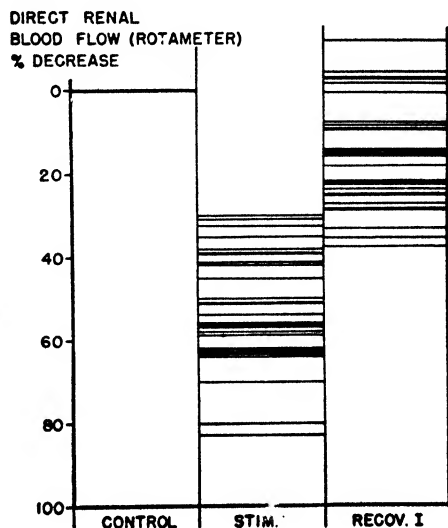
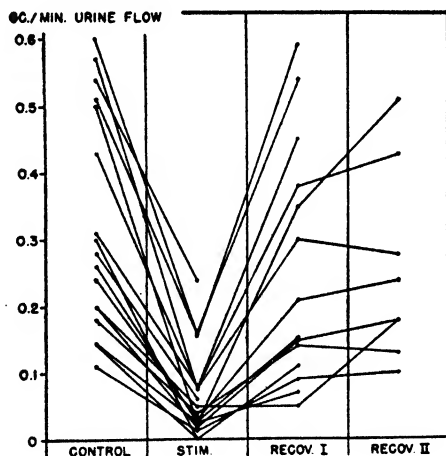


Fig. 3. EFFECT OF RENAL NERVE stimulation on urine flow (12 experiments).



By calculating blood flow according to the formula  $RBF = \frac{Cl_D}{1-Ht}$  the flow values indicated by the squares were obtained. These results were all lower than the corresponding directly measured mean rates of flow. After including in the formula the  $E\%_{OD}$  from plasma  $[RBF = \frac{Cl_D}{1-Ht} / E\%_{OD}]$  the calculated blood flows indicated by

the crosses were obtained. The fact that these values still deviated materially from the directly measured flows suggested that one of the factors in the latter equation was incorrect. If a significant diffusion of diodrast from the red cells to plasma had occurred in the renal vein blood sample, the extraction value obtained would be lower, and the calculated flow would be higher than the true values by a variable and unknown amount. Analysis of whole blood and plasma diodrast in systemic and renal venous samples demonstrated that a significant shift could have occurred in less than two minutes after samples were drawn and before centrifugation was complete. The amount of diodrast extracted from whole blood, expressed as per cent removed from

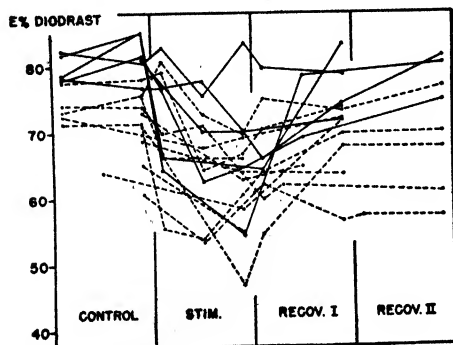


Fig. 4.  $E\%D$  FROM PLASMA during control, renal nerve stimulation and recovery. Solid lines indicate  $E\%D$  corrected for cell shift of D from cells to RV plasma (6 experiments); interrupted lines indicate uncorrected  $E\%D$  values (12 experiments).

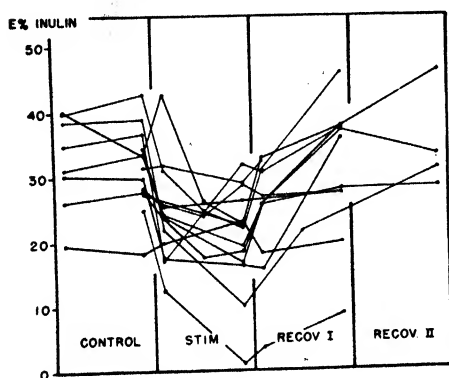


Fig. 5.  $E\%IN$  FROM PLASMA during control, renal nerve stimulation and recovery.

arterial plasma, was calculated as being the true plasma  $E\%$  for diodrast, corrected for shift from cells, the formula being:  $E\%_{\text{corr.}} =$

$$\frac{\text{sys. WB D mg.}\% \text{ minus RV WB D mg.}\%}{\text{sys. P D} \times (1 - \text{ave. sys. \& RV Ht})}$$

For the purpose of calculating only whole blood flow, arterial and renal venous whole blood analyses alone would be needed. Even though not more than 30 seconds elapsed from the drawing of samples to the time centrifugation was begun, the uncorrected  $E\%$  in 36 determinations (5 experiments) was 1.8 to 26.4 per cent lower

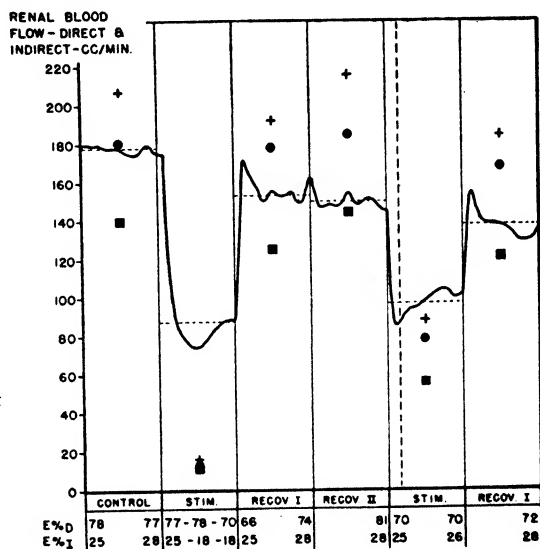
(av. 9.9%) than the corresponding values corrected for cell shift of diodrast using the above method (see example, table 1). With this correction the calculated flow closely approximated flow measured by the rotameter, but only during the control periods and before renal nerve stimulation had altered blood flow and urine flow (see closed circles, compare control and subsequent periods, fig. 6).

During the control periods the plasma level of diodrast, its concentration in the urine, the extraction percentage and urine flow were reasonably constant and the lag in the delivery of urine from the renal tubules to the pelvis of the kidney was essentially the same at the beginning and at the end of a given collection period. Under these conditions, which presumably existed only during the control periods, blood flow computed from clearance and corrected plasma D or whole blood D

Fig. 6. GRAPHIC SUMMARY of experiment shown in figure 1 with inclusion of additional periods of stimulation and recovery. Solid curve—reconstruction of direct RBF from original record. Horizontal interrupted lines indicate mean rate of direct RBF for each period. Squares—RBF calculated

as  $\frac{Cl_D \text{ from } P \left( \frac{UV}{P} \right)}{1 - Ht}$  Crosses.—  
RBF as  $\frac{Cl_D \text{ from } P/1 - Ht}{E\%_D \text{ from } P}$

Circles—RBF as  $\frac{Cl_D \text{ from } P/1 - Ht}{E\%_D \text{ from } P}$   
corrected for cell shift of D.  $E\%_D$  (corrected for cell shift) and  $E\%_{IN}$  given at bottom of each column.



extraction data might be expected to approximate the flow value indicated by the rotameter. In all of 8 experiments the calculated flow values for the control periods were 97 to 115 per cent (av. 104%) of the rotameter flow values. Similar agreement during control periods has been reported by Conn and Markley (12). However, during nerve stimulation the systemic plasma level of diodrast rose,  $E\%$  fell and its concentration in the intratubular urine presumably increased as urine flow diminished markedly. As a consequence a smaller proportion of a smaller amount of urine was collectable during the period even though the renal pelvis was washed with saline. In addition, the urine which was collected during the stimulation period included the 'lag urine' with a diodrast concentration of that secreted during the control period; but it did not include the urine of high diodrast concentration formed near the end of the stimulation period, which was the lag urine to be excreted into the renal pelvis during the following recovery period. The flows based upon clearance-extraction data were in good agreement with those obtained with the rotameter during control



periods, were much less than the actual flows during nerve stimulation when all urine formed was not available at the renal pelvis, and were greater than the actual flows during recovery when the previously formed urine was washed out. That the lag in the transport of urine to the renal pelvis was largely responsible for the differences receives support from the fact that when the effect of urine lag was minimized by considering 4 to 6 periods (including control, stimulation, and recovery periods)

TABLE 1. DISTRIBUTION OF DIODRAST, ITS DIFFUSION FROM RED CELLS, AND RESULTANT EFFECT ON EXTRACTION PER CENT DIODRAST

EX- TRACTION NO.	MG. % DIODRAST <sup>1</sup> IN SYSTEMIC:			SYSTEMIC HEMATOCRIT	MG. % DIODRAST IN RENAL VENOUS:			RV HEMATOCRIT	RV CELL: PLASMA D RATIO			(C) MINUS (H) <sup>4</sup>	$\frac{(K) \times (I)^5}{1 - (I)}$	(F) MINUS (L) <sup>6</sup>	$\frac{(F) - (M)^7}{(M)} \times 100$	$\frac{(A) - (F)^8}{(A)} \times 100$	$\frac{(A) - (M)^7}{(A)} \times 100$	$\frac{(F) - (O)^{10}}{(F)} \times 100$
	SYSTEMIC CELL: PLASMA D RATIO				RV CELL: PLASMA D RATIO													
	Plasma	Whole Blood	Cells <sup>2</sup>		Plasma	Whole Blood	Cells <sup>3</sup>		Plasma	Whole Blood	Cells <sup>3</sup>							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	
1	1.815	1.463	0.959	41.1	.528	.594	.632	.685	42.2	1.153	.274	.200	.394	51	67.3	78.3	14.0	
2	1.826	1.463	0.949	41.4	.520	.583	.643	.726	41.6	1.245	.223	.159	.424	38	68.1	76.8	11.3	
3	1.815	1.463	0.971	41.7	.535	.627	.643	.666	40.4	1.062	.305	.207	.420	49	65.4	76.8	14.8	
4	1.914	1.578	1.104	41.5	.577	.693	.704	.721	41.2	1.040	.383	.268	.425	63	63.8	77.8	17.9	
5	2.068	1.628	1.007	41.5	.487	.847	.781	.690	42.0	0.815	.317	.230	.617	37	59.0	70.2	15.8	
6	2.090	1.578	0.860	41.6	.411	.792	.775	.751	42.2	0.948	.190	.080	.712	11	62.1	65.9	4.7	
7	1.958	1.529	0.925	41.5	.472	.627	.676	.746	41.4	1.190	.179	.126	.501	25	68.0	74.4	8.6	
8	1.848	1.562	1.135	40.1	.614	.627	.665	.722	40.6	1.151	.413	.282	.345	82	66.1	81.3	18.7	
9	2.068	1.639	0.972	39.1	.470	.737	.759	.792	39.4	1.075	.180	.117	.620	19	64.4	70.0	8.0	
10	2.046	1.650	1.036	39.2	.506	.781	.781	.781	39.3	1.000	.255	.165	.616	27	61.8	69.9	11.5	
11	2.002	1.628	1.036	38.7	.517	.693	.748	.837	38.6	1.208	.190	.125	.568	22	65.4	71.6	13.7	

<sup>1</sup> Expressed as mg. % diodrast-iodine.

<sup>2</sup> By calculation  $B - [A(x - D)]/D = \text{mg. \% diodrast in systemic cells.}$

<sup>3</sup> By calculation  $G - [F(x - I)]/I = \text{mg. \% diodrast in renal vein cells.}$  <sup>4</sup> Shift of diodrast from cells, expressed as mg. diodrast/100 cc. of cells.

<sup>5</sup> Shift of diodrast to renal vein plasma, expressed as mg. diodrast/100 cc. renal vein plasma. <sup>6</sup> Corrected mg. % diodrast in renal vein plasma; mg. % found (by analysis) minus mg. % added to renal vein plasma by cell shift (calculated).

<sup>7</sup> Percentage renal vein plasma diodrast was increased by shift of diodrast from cells to plasma. <sup>8</sup> Uncorrected for cell shift. <sup>9</sup> Corrected for cell shift. <sup>10</sup> Percentage error of uncorrected extraction per cent diodrast due to cell shift.

These data were obtained from the experiment shown in figures 1 and 6.

as one long period the average calculated blood flow agreed with the average directly measured flow within +10 to -14 per cent (av. +1%) in all of 8 experiments.

Calculations of glomerular filtration rate and the clearance of inulin and diodrast would be expected to show comparable discrepancies for the same reasons as given above. As derived from the experiment illustrated in figures 1 and 6 the more directly measured and the computed values for these expressions are shown in table 2. The trends illustrated in this experiment were the same as those found in five similar experiments. During the control period, under reasonably constant conditions of equilibrium,  $GFR \text{ as } Cl_{IN}(UV/P)$  (period 1, col. 7) agreed with  $GFR \text{ as } RPF (RBF, \text{ rota. } \times 1-Ht) \times E\%_{OIN}$  (col. 8) and  $Cl_D(UV/P)$  (period 1, col. 4) agreed with  $Cl_D$  as

RPF (RBF, rota.  $\times$  1-Ht)  $\times$   $E\%_{OD}$  from P (corrected for cell shift) (col. 5). Following renal nerve stimulation (period 2) with accompanying reduction in urine flow and altered extractions, the values dependent upon urine collection were consequently low. During recovery (periods 3, 4) abnormally high values were obtained owing to the fact that the previously extracted substances were washed out with the returning urine flow. Again, if the deficit-excess 'artifacts' due to urine lag are smoothed out by regarding the entire experiment as one long period, the indirectly calculated values for total renal plasma flow, glomerular filtrate, and cc. of plasma cleared of D agree closely with the more directly determined values. (See table 2, bottom line. Compare col. 2 with 3, col. 7 with 8, and col. 4 with 5.)

As would be anticipated there were corresponding differences in the computed values for, and the directional changes in filtration fraction. Four expressions for FF, calculated with different combinations of derived values in the numerator and denominator (col. 10-13), may be compared with col. 9 in which  $E\%_{OIN}$  is regarded as the equivalent of the true FF. Although columns 10 and 12 gave close approximations to the actual FF in the control period, only  $E\%_{OIN}$  indicated the percentage of the plasma filtered<sup>5</sup> during renal nerve stimulation.

In 9 of the dogs India ink or trypan blue was injected caudally through a carotid artery into the aorta during unilateral renal artery-nerve stimulation of 1 to 10 minutes' duration and while renal blood flow was reduced to 40 to 60 per cent of the control flow. The kidneys were simultaneously excised within one half to one minute after beginning the injection. Diffuse distribution of dye was always present in the nonstimulated kidney while the stimulated one exhibited grossly on section either a uniform staining of greatly diminished intensity or slight staining throughout most of the kidney with several pyramidal shaped areas extending through the cortex and medulla which showed injection of moderate intensity. The pyramidal shape of the darker areas suggested that the latter may have been associated with nerves which had not been included in the bundle stimulated by the electrodes. In the palest areas of the stimulated kidney a low power lens revealed a sparse but even scattering of pale-dyed glomeruli through all of the cortex. In no instance was there a discernible difference between the amount of dye present in the juxtamedullary area and that in the more peripheral areas of the cortex.

#### DISCUSSION

Experiments have been presented which demonstrate that the extraction percentages of inulin and diodrast drop during the decrease in renal blood flow which occurs with acute stimulation of the renal nerves. The decreases in extraction percentages are reversible and return toward the control values after stimulation has been discontinued and blood flow has returned toward its control level.

Constriction of the afferent arterioles appears to be the primary effect of renal nerve stimulation since blood flow, filtration fraction ( $E\%_{OIN}$ ) and  $E\%_{OD}$  decrease concomitantly. If the formulae for calculating intrarenal resistance as devised by Lamport (16) and modified by Selkurt (17) are valid and applicable to the present

<sup>5</sup> It is assumed in this connection that inulin is 'filtered' in essentially the same concentration as it exists in the systemic plasma.

TABLE 2. CHANGES IN RENAL PLASMA FLOW, EXTRACTION, AND CLEARANCE OF INULIN AND DIODRAST, AND FILTRATION FRACTION WITH RENAL NERVE STIMULATION

URINE FLOW cc/min.	RENAL PLASMA FLOW CC/MIN. AS:			CLEARANCE OF DIODRAST CC/MIN. AS:		GLOMERULAR FILTRATION RATE CC/MIN. AS:		FILTRATION FRACTION AS:					
	RPF (rotameter) X 1 minus Ht	Cl <sub>b</sub> E% D (corrected)	UV P	RPF (rotameter) X E% D (corrected)	E% D FROM PLASMA (CORRECTED)	Cl <sub>N</sub> UV P	RPF (rotameter) X E% IN	E % IN (E <sub>RM</sub> )	Cl <sub>N</sub> RPF (rotameter)	E% IN RPF (rotameter)	Cl <sub>N</sub> E% D (corrected)	Cl <sub>N</sub> Cl <sub>b</sub>	
Column→	1	2	3	4	5	6	7	8	9	10	11	12	13
Period ↓ I Cont.					(2) X (6)			(2) X (9)		(7) (2)	(9) X (2) (4)	(7) (4)/(6)	(7) (4)
	0.5	105	106	82.4	82.2 80.6	78.3 76.8	25.5	25.8 28.9	24.6 27.5	.243 .238	.313 .351	.247	.309
2 Stim.													
	0.02	51	8.3	6.2	39.2 35.8	76.8 77.8 70.2	1.9	12.9 9.0 8.9	25.3 17.7 17.6	.037	1.66 1.45 1.44	.230 .239 .215	.306
3 Rec. I													
	0.35	90	104	72.9	59.3 67.0	65.9 70.1 74.4	23.7	22.3 25.3	24.8 26.5 28.1	.263	.306 .347	.214 .228 .242	.325
4 Rec. II													
	0.51	89	109.3	85.1	69.2 81.3	77.8	29.4	24.8 27.7	.330	.291	.269	.345	
5 Stim.													
	0.16	59	48.3	33.8	41.3 41.2	70.0 69.9	11.4	14.8 15.3	25.0 26.0	.192	.438 .453	.235	.336

6 Rec.	0.54	84	103	73.8	60.1	71.6	24.7	23.1	27.5	.204	.313	.240	.335
Total plasma flow, clearance, and filtration rate over six periods													
		6222 cc.	6169 cc.	4575 cc.	4608 cc.		1810 cc. Av. 22.9 cc/min.	1614 cc. Av. 20.7 cc/min.					

experiments, the computed afferent resistance increased an average of 158 per cent while efferent resistance decreased an average of 60 per cent.

The simultaneous reduction in urine flow makes it impossible to measure quantitatively the change in filtration and secretion by the usual clearance methods. The inaccuracy is particularly great in the present experiments in which no diuretic was administered and in which the collection periods were of only 10 to 20 minutes' duration. By multiplying plasma flow (rotameter)  $\times E\%_{\text{OIN}}$  and  $E\%_{\text{OD}}$ , it was possible to determine the diminished but still appreciable amounts of glomerular filtrate formed and diodrast secreted during nerve stimulation when the flow of urine almost ceased. The actual clearance of inulin and diodrast decreased to average values of 30 and 39 per cent respectively of their control values. Depending upon urine flow the apparent clearances (UV/P) in different experiments ranged from 1 to 70 per cent of the actual values.

The observation that renal nerve stimulation causes decreases in RPF and GFR in acute experiments confirms the findings obtained by Kubicek and Kottke (2) during the early phases of their chronic stimulation experiments. From a quantitative standpoint it is likely that their clearance figures were low, as are those reported here, because of decreased urine flow. If the extraction of diodrast and *p*-amino-hippurate are similarly decreased with acute nerve stimulation, their apparent RPF values (using PAH) were probably further diminished because of a simultaneous decrease in extraction.

It is possible to infer from the present experiments whether a shift of D from cells to plasma occurs *in vivo* during passage through the kidney and/or *in vitro* after renal venous blood is drawn. White (18, 19) has presented experimental data from which he concluded that D shifts from cells to plasma and is then secreted into the urine before the blood reaches the renal vein. If a significant amount of D were to be 'cleared' from cells and secreted into the urine in this way the clearance values based upon arterial plasma concentration would be erroneously high. During the control period of the present experiments the amount of D appearing in the urine divided by arterial plasma concentration gave clearance values which were not significantly differ-

ent from the clearances determined by multiplying 'directly' measured RPF (RBF, rota.  $\times$  1-Ht)  $\times$   $E\%_{OD}$  from plasma, corrected for the cell shift of diodrast, regardless of where it occurred (table 2, col. 4 and 5 for period 1, and bottom line [total]). Consequently the D which appeared in the urine must have been cleared almost exclusively if not entirely from the plasma fraction with little or no *in vivo* extraction from red cells. Using  $RPF = Cl_D/E\%_{OD}(\text{uncorrected})$  it appears that the abnormally high RPF values obtained by White (18) are more likely to be explained by the  $E\%_{OD}$  being too low (because of cell shift of D after drawing venous sample) rather than by  $Cl_D$  being too high (because of partial clearance of cell D into urine).<sup>6</sup> In the control periods of the experiments reported here (see example, fig. 6) the use of  $E\%_{OD}$ , uncorrected for cell shift, gave calculated RPF values which averaged 112 per cent of the actual RPF, which figure compares with the 113 per cent given by White.

Under the conditions of the acute experiments reported here there was found no evidence of an intrarenal diversion or 'shunting' of blood during direct stimulation of the renal nerves in the anesthetized dog. If blood were to be by-passed around glomeruli, or tubules, or both, theoretically a decrease in  $E\%_{OIN}$  or  $E\%_{OD}$  or both would occur. However, a drop in extraction would not necessarily indicate the presence of a shunt since extractions would also be expected to decrease under circumstances which would lessen the filtering ability of the glomeruli or the secretory ability of the tubules. Although it seems more probable that, through the latter mechanism, afferent constriction and relative ischemia are the factors responsible for the observed decreases in  $E\%_{OIN}$  and  $E\%_{OD}$ , it is not possible to determine from the extraction data what portion of the decrease, if any, is attributable to by-passing of blood around some of the glomeruli or tubules. If this type of shunting existed during nerve stimulation in the present experiments, any perfusion of non-extracting tissue must have occurred in spite of the decrease in total blood flow. The magnitude and distribution of such shunting would not be revealed by a dye injection method although the latter did serve to demonstrate that anatomically the juxtamedullary area was rendered no less ischemic than the more superficial layers of the cortex.

#### SUMMARY

Simultaneous determinations of extraction and excretion of inulin and diodrast, and unilateral renal venous blood flow measurements (rotameter) were made in anesthetized dogs. During control periods the directly measured and calculated (Fick) blood flow values agreed closely.

The renal nerves to the ipsilateral kidney were stimulated with low frequency alternating current, and with the strength of stimulus used the following average decreases were observed: direct renal blood flow, 53 per cent; extraction of inulin (and filtration fraction) 38 per cent; extraction of diodrast, 17 per cent; urine flow, 81 per cent. Glomerular filtration and tubular secretion continued at a reduced rate although urine flow was greatly diminished. Because of the reduction in urine formation during nerve stimulation, inulin and diodrast clearances and calculated renal

<sup>6</sup> This concept is in agreement with that suggested by Phillips, *et al.* (20) in considering the cell shift of PAH.

blood flow were from 1 to 70 per cent of their true values and all exceeded the latter during recovery with the return of urine flow. The factors limiting the usefulness of clearance methods in this type of study are discussed.

A significant *in vitro* diffusion of diodrast from red cells to plasma occurred within 2 minutes after drawing samples. Using whole blood analyses corrected extraction values were obtained. Without correction for diffusion the extraction percentage of diodrast would have been 2 to 26 per cent lower and calculated RBF 3 to 55 per cent higher than the actual values.

Constriction of the afferent arterioles appeared to be the primary effect of renal nerve stimulation. There was little or no accompanying change in blood pressure.

Dye injected into the aorta during unilateral nerve stimulation caused intense staining of the unstimulated kidney and a pale but diffuse staining of the stimulated kidney. In the experiments performed there was no evidence to suggest that the stimulation caused a shunting of blood from the outer renal cortex to the juxta-medullary areas.

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## MEASUREMENT AND SIGNIFICANCE OF URINARY APPEARANCE TIME IN THE DOG

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THE expression 'appearance time' signifies the time interval from the moment of intravenous injection of a test substance to its first detectable appearance in the urinary bladder. This period is chiefly made up of 1) circulation time from the site of injection to the glomerular or tubular vessels and 2) the time required for the substance to travel from its site of excretion down the tubules, through the pelvis and ureters and into the bladder. The purpose of this study is to determine the appearance times of substances used in renal physiology and to define the significance of these measurements in renal clearance studies. A review of the pertinent literature is included in order to resolve existing confusion in concept and terminology.

### MATERIALS AND METHODS

*Operative Procedure.* Two trained female dogs were used: dog 1, weighing 6.5 kg. and dog 2, 18 kg. Under light sodium pentobarbital anesthesia, the vesical trigone with the adjacent floor of the bladder was exteriorized, producing an artificial extrophy of the bladder (1, 2). The initial experiments were started 10 days postoperatively. During all observations the dog was lightly restrained upon its back on an animal board.

*Urine Collection.* Urine was collected as it emerged from one or both ureters into a plastic centrifuge tube by means of a suction cup applied to the bladder mucosa. The urine was allowed to fill the cup, and a urine collection period started as the first drop of urine appeared in the collecting tube. After an accurately timed period of 1 to 3 minutes, the collection of urine was stopped by turning a stopcock between the collecting tube and suction pump, thus instantly terminating the collection period. Immediately following each measurement of appearance time the rate of urine flow was determined by collection for a known interval.

*Appearance Time Determination.* The appearance times of the following substances were determined after injection in a jugular vein:

*Indigo carmine:* dose, 2 cc. of an 0.8 per cent solution. The first detectable appearance of bluish urine from either ureter was taken as the appearance time.

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*Phenolsulphonphthalein*: dose, 1 cc. (6 mg.). The bladder mucosa was soaked with a solution of sodium bicarbonate and a tablet of sodium bicarbonate was held directly over each ureteral orifice. The first reddening of the urine upon contact with the sodium bicarbonate was noted as the appearance time.

*p-Aminohippurate*: dose, 0.5 to 1 cc. of 25 per cent solution. Sixty seconds after the injection of PAH, an absorbent asbestos<sup>6</sup> pledget was dabbed repeatedly over the ureteral orifices for 5 seconds to collect the bulk of the urine appearing during this interval. The pledget was then dropped into an analytical tube, and a fresh pledget substituted. The same procedure was repeated every 5 seconds. Each pledget was then analyzed for the presence of PAH by the technic of Bratton and Marshall (3). The appearance time, within 5 seconds, was easily identified by the first tube showing the presence of PAH.

*Inulin*: dose, 4 to 5 cc. of 10 per cent solution. The same procedure was used as in the determination of PAH appearance time. In the analysis, (4) 3 cc. of diphenylamine reagent were added to each of the tubes, which were then immersed in boiling water for 10 minutes. The blue color which appeared in the first tube containing a trace of inulin was unmistakable.

*Sodium ferrocyanide*: dose, 1 cc. of 25 per cent solution. Pledgets of cotton were used instead of asbestos to absorb the urine during each 5-second interval. Three cc. of a concentrated solution of ferric sulphate were then added to each of the tubes. The first tube developing a bluish discoloration of the cotton pledget revealed the appearance time.

All intervals were timed by means of a stop-watch.<sup>7</sup> The end point in each of the above reactions was sharp and well defined. Indigo carmine, phenolsulphonphthalein and PAH are excreted by the tubules in addition to being filtered; inulin and sodium ferrocyanide are excreted solely by filtration. Appearance time determinations were made at varying rates of urine flow. Antidiuresis was induced by water deprivation, and diuresis by either the oral administration of water or by the intravenous injection of 60 to 150 cc. of 25 per cent solution of mannitol.

## RESULTS

One hundred and sixteen measurements of appearance time were made, 78 in *dog 1* and 38 in *dog 2*. Data obtained from *dog 1* (6.5 kg.) showing the relation of 'tubular' appearance time to urine flow, are illustrated in figure 1A, and data for 'glomerular' substances in figure 1B. There appears to be no essential difference in the excretion of the two types of substances. The appearance time of all substances decreases abruptly from 130 seconds at low urine flows to 80 seconds as the urine flow reaches 0.5 cc/minute; this interval then remains at 80 seconds until the urine flow increases to 2.0 to 2.5 cc/minute, when it increases to about 100 seconds. This plateau of 100 seconds is maintained with little scatter until a flow of about 6 cc/minute is reached, when the appearance time decreases again, the lowest figure obtained being 68 seconds at a urine flow of 8 cc/minute.

Similar observations on *dog 2* (18 kg.) are shown in figure 2. The appearance time of 'tubular' and 'glomerular' substances in relation to urine flow forms a curve

<sup>6</sup>Cotton, sponge, and filter paper were tried in control studies but were discarded because of the high blanks for PAH and inulin.



of essentially the same pattern as that obtained in the smaller dog. The appearance time decreases from about 180 seconds at low urine flows to 80 seconds at a flow of 1 to 3 cc/minute and at higher flows it increases to 100 seconds. This plateau of 100 seconds is maintained until a flow of about 10 cc/minute is reached, when the interval declines, the lowest figure obtained being 70 seconds at a maximum flow of approximately 13 cc/minute.

A comparison of the appearance time in these 2 dogs shows that, in spite of the marked difference in size of the 2 animals, the same general pattern is preserved and the values fall within the same range. However, the characteristic changes in the contour of the curve in the smaller dog occur at lower urine flows than in the larger animal.

#### DISCUSSION

Our observations indicate that, except during oliguria, the first appearance time remains practically constant in spite of wide changes in the rate of urine flow.

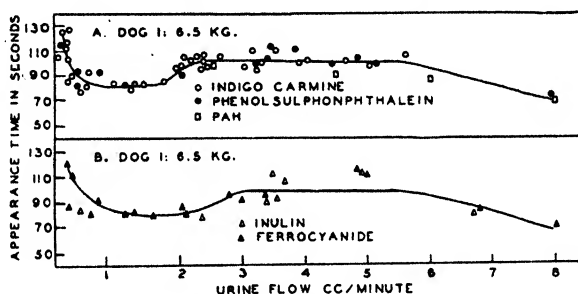


Fig. 1. RELATION between appearance time and urine flow in a 6.5-kg. dog: A, for 'tubular' substances, and B, 'glomerular' substances.

If the renal tubules and collecting ducts are conceived to be a system of rigid tubes, any increase in urine flow must bring about an increase in the linear velocity of urine, and any substance entering the proximal end will travel faster and appear sooner from the distal end, and appearance time will vary inversely with urine flow.

Our data do not conform with this view except at relatively low urine flows. As the urine flow increases above 1.5 cc/minute in the smaller dog, or above 3.0 cc/minute in the larger dog, the appearance time paradoxically increases from 80 to 100 seconds, and remains constant at the higher figure, despite a further 3-fold increase in urine flow.

It appears that, at urine flows above an appearance time of 80 seconds, the tubules cease to act as rigid conduits and are passively dilated by the increasing urine volume, thus offsetting the simple mechanical effect of increasing flow. The abrupt increase in appearance time to 100 seconds indicates that the velocity of the urine in the tubules has decreased, implying sudden dilatation out of proportion to increased urine volume, and the maintenance of a constant appearance time of 100 seconds at higher urine flows implies a passive increase in cross-sectional diameter of the tubules such as exactly to balance the further increase in urine volume.

At extremes of osmotic diuresis, the appearance time again decreases with increasing urine flow, possibly because the tubules have reached the limits of distensibility. Another factor that may contribute to this reduction in appearance time at extremely high rates of urine flow is the fact that under these conditions the urine issues from the ureters in an almost continuous stream, rather than in intermittent peristaltic waves.

The evidence cited above that the tubules dilate during diuresis is supported by histologic evidence. Brodie and Mackenzie (5, 6) measured the diameter of the tubules before and during diuresis in cats and dogs and found them to increase during diuresis. The average diameter of the lumen of the proximal tube before and after diuresis was 0 and  $19.4 \mu$ , and of the distal tubule, 11 and  $21.8 \mu$ . They observed that a kidney at the height of diuresis is hard, and the capsule tensely distended. Volhard (7) similarly records that there is tubular dilatation and a slight flattening of the epithelium at the height of diuresis.

It is possible that the pelvis as well as the tubules participate in this dilatation. Bojesen (8) has concluded from direct measurements of the pelvic volume of dogs in different states of diuresis that this volume increases with increasing urine flow. Although his conclusion is plausible, it is not well substantiated by his recorded evidence because of wide scatter in the data.

The determination of appearance time has long been used as a gross test of renal functional efficiency (9), and in recent years it has acquired interest for investigators concerned with clearance determinations. In 1938, Smith, Goldring and Chasis (10) measured the appearance time of phenolsulphonylthalein eighteen times in 10 subjects, and observed that the determinations varied from 120 seconds at a urine flow of 20 cc/minute to 200 seconds at a urine flow of 1 cc/minute. They concluded that at urine flows of 6 to 2 cc/minute, the appearance time averaged 150 seconds. Instead of the terms 'appearance time', they labeled the measurement 'minimal excretion time'. These studies were initiated by the fact that, in clearance studies, a particular sample of urine is formed from blood not of the composition possessed by simultaneous systemic venous blood but of the composition existing some seconds before the urine collection period. This interval, which they designated as 'delay time', is made up of: 1) circulation time from a peripheral vein to the renal capillaries, 2) time required for the substance to pass from the glomerulus or tubular cell into the tubular lumen, and 3) dead space time, i.e. the time required for the urine to pass down the tubules, through the pelvis and ureters, and into the bladder. Thus defined, 'delay time' is identical with first appearance time. Subsequently, whenever renal clearances were determined, corrections for delay time were made by interpolating the blood curve not to the middle of the urine collection period but to a point 150 seconds before the mid-point.

The measurement of 'delay time' becomes important in clearance studies whenever the plasma concentration of the test substance is changing. The problem of relating the plasma concentration to a particular sample of urine is, however, a complicated one. The length and diameter of individual nephrons vary and it may be accepted that the velocity of urine flow in each may also vary. Consequently, a substance filtered through different glomeruli simultaneously will not appear in the

bladder at precisely the same moment. Conversely, a particular sample of urine entering the bladder is formed from glomerular filtrate that was formed in various glomeruli at different times. Furthermore, in the presence of a rising concentration in the plasma, it is to be expected that a urine sample in its transit to the bladder will be subjected to the diluting influence of the urine formed before it and to the concentrating influence of the urine formed after it, so that by the time it reaches the bladder its composition is not related to a specific plasma concentration; rather, it has been formed from different concentrations of plasma passing the glomeruli at different moments. These variations are grossly averaged by the collection of blood and urine over protracted periods. Consequently, delay time, as applied to clearance determinations, is approximately equal to first appearance time.

Gaudino and Levitt (11), in studying inulin space as a measure of extracellular fluid volume, attempted to determine the quantity of inulin contained within that portion of the urinary tract between Bowman's capsule and the bladder (dead space) after equilibrium distribution of inulin throughout the extracellular fluid had been

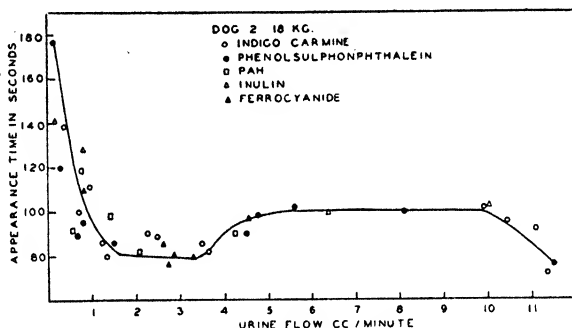


Fig. 2. RELATION between appearance time and urine flow in an 18-kg. dog.

obtained. Using a technically difficult and, we believe, potentially inaccurate method, they measured a time interval which they called 'delay time', but which is in effect an approximation of first appearance time. Contrary to the observations reported here, they concluded that delay time (or, properly, first appearance time) is inversely proportional to urine flow. It is pertinent to mention the possible sources of error in their method: 1) potential inaccuracy of bladder washouts within the short space of 2 minutes; 2) inadequacy of the analytical method to detect small differences in the concentration of inulin;<sup>6</sup> and 3) possible misinterpretations of relatively few observations secured from different animals. These investigators multiplied this time interval (expressed in minutes) by the rate of excretion (mg/min.) in order to obtain the quantity of inulin contained in the dead space; this calculation would be valid if the volume of the dead space (tubules, pelvis and ureters) remained constant, but our present evidence indicates that the dead space increases almost in proportion to urine flow.

Bojesen (8) concludes that dead-space error (delay time) cannot be reduced

<sup>6</sup> The accepted chemical error in the analysis of inulin is  $\pm 5$  per cent.

by diuresis, a conclusion that is in agreement with our present observations. Brun, Hilden, and Raaschou (12), from a study of the rate of excretion of thiosulphate after establishment of a constant plasma level in man, arrive at a mean delay time of 5 minutes for urine flows of 2 to 5 cc/minute. The experimental data cited are, we believe, insufficient to establish this point; in particular it may be noted that the procedure of collecting one minute urine samples from the bladder is subject to considerable error.

Our study has shown that, during moderate diuresis, the appearance time of substances commonly used in renal physiology averages 100 seconds in dogs, irrespective of size. It is suggested, therefore, that during clearance studies in dogs a condition of moderate diuresis be maintained and the plasma concentration corresponding to a given urine sample be determined by interpolating the plasma concentration to a point 100 seconds before the mid-point of the urine collection period. It would seem, on the basis of comparative body weight, that the use of 150 seconds for correcting clearances in human subjects, as advocated by Smith and his coworkers, is valid if a state of moderate diuresis is maintained.

Michie and Michie (13) have shown by ureteral catheterization in man that, in the normal kidney, after the rapid establishment of a nearly constant plasma level and at urine flows of 1 to 4 cc/minute per kidney, approximately 20 to 30 minutes are required before the urine comes into equilibrium with the plasma in the excretion of inulin, mannitol, thiosulphate, PAH and phenol red. This observation is in agreement with an average first appearance time in man of 150 seconds, as is demonstrable by the following theoretical analysis.

In a series of tubes (tubules, pelvis and ureters), differing in physical characteristics but emptying into a common outlet (bladder), several considerations are worthy of note. When a particular substance enters the proximal ends of the tubules simultaneously, and the subsequent rate of entry remains constant, the time of appearance of the various particles of this substance at the outlet will vary. Equilibrium in the system, i.e. when the rate of entry equals the rate of exit, will occur when, and only when, the particles requiring the longest time to traverse the system emerge from the distal end; this is 'equilibrium time.' Our results reported above apply only to the particles exhibiting the shortest appearance time.

One of the authors (D.G.) utilizing the implications indicated above, by use of distribution curves for appearance times and a series of summations and integrations has arrived at the following mathematical formulation for equilibrium time based on

appearance time:  $T_e = \frac{t_a}{\sqrt{(1 - \sigma)^n}}$  in which  $T_e$  is equilibrium time,  $t_a$  is appearance time,  $\sigma$  is the fraction of equilibrium achieved, and  $n$  is a numerical parameter measuring the law of distribution of velocities which for laminar flows equals unity.<sup>7</sup> Utilizing this equation and assuming an appearance time ( $t_a$ ) of 2.5 minutes (10), in order to reach 99 per cent equilibrium ( $\sigma$ ), equilibrium time ( $T_e$ ) would be 25 minutes.  $T_e = \frac{2.5}{\sqrt{0.01}} = 25$ . This figure is in good agreement with the experimental data of Michie and Michie.

<sup>7</sup> There is every reason to believe that all flows through the kidney are laminar.

## SUMMARY

Measurements of appearance time of substances used in renal physiology were made at varying urine flows in 2 dogs of different sizes. Appearance time is independent of urine flow during moderate diuresis, increasing only at very low urine flows and decreasing moderately during extreme diuresis. The relative constancy of appearance time at urine flows between extreme oliguria and extreme diuresis is attributed to tubular dilatation as the urine flow increases. During moderate diuresis in the dog, appearance time averaged 100 seconds. This value can be utilized in making delay time corrections in clearance studies. On the basis of these studies, the commonly used delay time of 150 seconds in clearance studies in man appears to be valid as a correction for moderate changes in plasma concentration of test substances during moderate diuresis. Taking the minimal appearance time in man as 150 seconds, it is shown theoretically that, after an instantaneous change in plasma concentration of a solute, approximately 25 minutes will be required for the urine again to come into equilibrium with the plasma, a deduction conforming with previously recorded observations.

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# CLEARANCES OF SOME PROTEINS BY THE DOG KIDNEY<sup>1</sup>

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QUANTITATIVE studies of the filtration of proteins by the kidney have been retarded because of the difficulty of assaying specifically for a given protein in the presence of urine or plasma proteins. Recently techniques have been developed for the immunochemical analysis for the egg white proteins, conalbumin (1), ovalbumin (1), ovomucoid (2), and lysozyme (3), and for  $\beta$ -lactoglobulin (4) in relatively complex biological fluids. With the exception of conalbumin these proteins either fall in the same size range or are identical with those shown previously by Bott and Richards (5) and by Bayliss *et al.* (6) to pass through the glomerular membrane. A study has been made of their clearance by the kidney of the dog. The results support the conclusion of previous workers that considerations other than the size and shape of the protein molecule are factors in the rate at which proteins are eliminated by the kidney. It has been observed that hemoglobin is excreted only after its concentration reaches a threshold value (6-8). This phenomenon, which is presumably due to tubular reabsorption of protein, may be involved in the elimination of the proteins studied here. Furthermore, evidence is presented in this communication that interaction of the injected proteins with the serum proteins affects their rate of excretion.

## METHODS

Some pertinent physical properties of the proteins used in this investigation are given in table 1. Conalbumin was prepared by the method of Bain and Deutsch (9). Ovalbumin was separated by a procedure involving precipitation with ethanol and was 98 per cent pure by electrophoresis. Lysozyme, isolated according to the directions of Alderton and Fevold (10), was recrystallized five times. Ovomucoid was prepared by the method of Fredericq and Deutsch (11).  $\beta$ -lactoglobulin was separated by the procedure of Cecil and Ogston (12) and recrystallized four times. The antisera to these proteins were either identical or similar to those employed in previous studies (1-4).

Trained female dogs weighing from 7 to 11 kg. were used. The animals were given 30 mg. of creatinine and 40 ml. of water/kg. by stomach tube from 45 to 60 minutes prior to any period during which the clearance of a protein was studied. The proteins were administered by intravenous injections of solutions made up in water

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and adjusted to pH 7.0 to 7.5. Because of the possibility of deleterious effects resulting from sensitization to foreign proteins only one experiment with each protein was carried out with each dog. Three or more days were allowed to elapse between each experiment with a given dog. This period appears to be of sufficient duration to permit the elimination of the major portion of the administered ovalbumin, the largest protein molecule studied (5, 13). In one group of experiments a relatively constant level of protein in the plasma was maintained by an initial injection of protein followed by infusion of the protein at a uniform rate throughout the experimental period. Clearances were determined during 3 consecutive 30-minute intervals. Samples of blood were taken from the femoral artery or vein at the mid-point of each interval. In a second group the initial concentration of protein in the plasma was high and the level was allowed to fall during the course of the experiment. This result was accomplished by injecting a relatively large amount of protein and omitting the infusion. In a third group the initial injection was omitted and a relatively concentrated solution of protein infused so that the level of protein in the plasma rose during the experiment. In the experiments falling into the latter two classifications,

TABLE 1. SOME PHYSICAL PROPERTIES OF THE PROTEINS USED FOR KIDNEY CLEARANCE STUDIES

PROTEIN	MOLECULAR WEIGHT	ISOELECTRIC POINT	AXIAL RATIO
Conalbumin	87,000	6.1	5
Ovalbumin	44,000	4.6 <sup>1</sup>	4
		4.7 <sup>1</sup>	
$\beta$ -lactoglobulin	35,000	5.2	5
Ovomucoid	27,000	3.9	8
Lysozyme	17,200	11.3	4

<sup>1</sup> The isoelectric points of the A<sub>1</sub> and A<sub>2</sub> components, respectively.

clearances were determined for 10-minute intervals. The first period was started 10 to 15 minutes after the initial injection, or the start of the infusion, and 20 minutes were allowed to elapse between each period. Samples of blood were again taken at the mid-point of each period.

Urine was collected by means of a urethral catheter. The bladder was rinsed three times with saline just prior to and at the end of each clearance period. The washings at the end of a period were added to the urine obtained during that interval.

The blood serum and urine samples were assayed for creatinine by the alkaline picrate method (14) and for the protein under consideration by the immunochemical methods previously described (1-4). The clearance of the protein for a given period was compared with the clearance of creatinine for the same period. The total urinary protein was determined from the nitrogen which could be precipitated by 5 per cent trichloroacetic acid. Ovomucoid cannot be determined by this method (11).

## RESULTS

Preliminary experiments established that the proteins employed could be quantitatively estimated in the presence of both serum and urine of dogs by the im-

munochemical method. With the exception of lysozyme the i.v. administration of the proteins used produced no observable effects. Three of 6 dogs showed marked symptoms upon the injection of lysozyme. These consisted of edema in the tissues about the eyes and lips and of an erythematous, vesicular rash on the abdominal surface. Only one of the 6 dogs, however, showed skin sensitization to lysozyme from 3 to 8 weeks after i.v. administration of this protein. Two of the dogs used to study the clearance of lysozyme were among those which did not show any visible reaction and the other was one which, in contrast to the other sensitive animals, maintained a fairly constant filtration rate during the experiment.

The experimental results of the studies of the clearance of the various proteins are discussed individually.

*Conalbumin.* This protein was not present in the urine in an amount sufficient to be detectable when the serum concentration was approximately 50 mg. per cent. Under the conditions of the test employed a clearance of 0.1 per cent of that of the creatinine could have been detected.

*Ovalbumin.* The data for the clearance of this as well as the other proteins are shown in table 2. In the experiment with *dog 3*, in which the concentration was maintained constant, the clearance was essentially the same over the 3 periods studied. On the assumption that the difference in clearance between *dogs 2* and *3* was due to the difference in the concentration of ovalbumin in the two cases, experiments were performed with *dogs 5* and *1* in which the concentration of ovalbumin was allowed either to increase or decrease markedly during the course of an experiment. As was anticipated, the clearance was greatest when the concentration of protein in the serum was highest.

*$\beta$ -lactoglobulin.* This protein gave very anomalous results. The clearance of it was less in the third period than in the first, whether the serum level of  $\beta$ -lactoglobulin was constant (*dog 3*) or increasing (*dog 4*) or decreasing (*dog 1*) during the course of an experiment. The one exception occurred in the experiment with *dog 2*. Here the clearance did increase with increase in the serum concentration, but in this case the serum concentration was very low. It can be seen from table 2 that, in contrast to ovomucoid and ovalbumin,  $\beta$ -lactoglobulin is lost very rapidly from the plasma. It is also cleared more rapidly than the other proteins when the serum concentration is relatively high.

*Ovomucoid.* The results for this protein are similar to those obtained with ovalbumin. At comparable serum concentrations it shows approximately the same clearance as ovalbumin. In the experiment with *dog 5* the clearance decreased with decrease in the level of ovomucoid in the serum. Because of lack of antiserum it was not possible to perform an experiment in the course of which the serum concentration was increased.

*Lysozyme.* The clearance of this protein, the smallest molecule studied, was not so great as might have been expected from the results obtained with ovalbumin and  $\beta$ -lactoglobulin. Moreover, its clearance appears to depend upon its serum concentration. These results may possibly be explained by the following consideration. Lysozyme is known to form complexes with other proteins (15, 16). It was possible that it combined with the serum proteins of the dog. If this were the case, one should probably use the concentration of free lysozyme in the serum rather than the total



concentration in order to calculate the percentage of the protein cleared. An estimate of the magnitude of this effect was obtained by the following 2 experiments, both of

TABLE 2. CLEARANCE STUDIES OF VARIOUS PROTEINS

PROTEIN	DOG	IN- ITAL IN- JECT.	IN- FU- SION RATE	FILT. RATE <sup>1</sup>	SERUM CONC.	PROTEIN CLEAR. CREATININE CLEAR. $\times 100$	PROTEIN	DOG	INITIAL INJE- CTION	IN- FU- SION RATE	FILT. RATE <sup>1</sup>	SERUM CONC.	PROTEIN CLEAR. CREATININE CLEAR. $\times 100$
	no.	mg.	mg./ min.	ml./ min.	mg. %			no.	mg.	mg./ min.	ml./ min.	mg. %	
Ovalbumin	2	100	1.1	48	17	13	Ovomucoid	1	100	0.5	39	16	10
				50	13	12					37	13	9
				46	11	10					35	12	10
	3	300	3.3	28	46	19		2	125	1.4	36	14	13
				27	43	20					38	11	11
				26	43	18					38	11	10
	5	0.10		24	34	15		3	100	1.7	32	14	6.2
				26	71	20					32	13	6.9
				26	94	21					31	13	6.8
	1	1000	0	35	115	21		5	1000	0	24	117	18
				30	60	19					21	76	15
				33	28	14					24	49	13
$\beta$ -lactoglobulin	2	150	1.7	42	4.3	8	Lysozyme	3	100	2.8	27	9.4	21
				42	4.6	12					24	11.2	24
				38	14	14					22	12.5	26
	3	300	6.7	28	24	51		2	175	2.0	35	10.1	33
				29	24	23					37	8.6	22
				33	24	29					31	8.2	24
	4	0.15		38	22	35	Ovalbumin + Ovomu- coid	1	165	0	34	7.5	42
				37	39	32					30	4.1	27
				42	46	30					28	2.9	20
	1	1000	0	27	44	68		4	200 <sup>2</sup>	2.5	42	27	16
				26	14	36					40	25	15
				28	4.7	6					39 <sup>3</sup>	24	15
											36	28	17
											34	28	20
									200 <sup>4</sup>	3.0	39 <sup>3</sup>	25	13
											36	25	13
											34	26	15

<sup>1</sup> Based on creatinine clearance. <sup>2</sup> Ovalbumin. <sup>3</sup> Beginning of ovomucoid clearance periods.

<sup>4</sup> Ovomucoid.

which depended upon the fact that lysozyme will diffuse slowly through ordinary Visking tubing (3, 17). In the first, dog serum in saline-borate buffer, pH 7.4, containing 6 mg. per cent of lysozyme, was dialyzed against 2 volumes of the same buffer

containing the same concentration of lysozyme. After 6 days at 1° to 2° the serum and dialysate were analyzed for lysozyme. Its concentration in the serum was 8.5 and in the dialysate 5.3 mg. per cent. The ability of the lysozyme to pass into the dialysis bag against an apparent concentration gradient strongly suggests that the dog serum proteins are binding a portion of the lysozyme, in this case approximately 38 per cent. In the second experiment lysozyme solutions of varying concentration, in the presence and absence of dog serum, were subjected to ultrafiltration through Visking membranes under the same conditions. It can be seen from table 3 that the presence of dog serum decreased the amount of lysozyme filtered. If, as the data from these *in vitro* experiments indicate, the concentration of free lysozyme is only 50 to 75 per cent of the total concentration of this protein in the serum, the true values for its clearance should be approximately double those given in table 2.

*Simultaneous Clearance of Two Proteins.* Since the concentration of a protein in the plasma appeared to affect its clearance, it was decided to determine the effect of a second protein on the clearance of the protein which was injected initially. For this purpose ovalbumin and ovomucoid were selected. The clearance of ovalbumin was determined for 2 successive 20-minute intervals. Ovomuroid was then injected, the infusion of both proteins continued, and their clearances followed for 3 more successive 20-minute intervals. It can be seen from table 2 that ovomucoid tended to increase the clearance of ovalbumin. The magnitude of the increase is what might have been expected had the ovomucoid been ovalbumin, i.e. had the serum ovalbumin level been essentially doubled.

#### DISCUSSION

It was hoped initially that the size and shape of the protein molecules studied could be employed to explain their clearances. To some extent this is possible. Lysozyme, even neglecting the consideration of binding, is cleared more rapidly than both ovalbumin and ovomucoid at similar serum levels. While the molecular weight of ovalbumin is greater than that of ovomucoid, the greater asymmetry of the ovomucoid molecule may explain the observation that ovalbumin and ovomucoid are cleared to approximately the same extent. Conalbumin appears to be beyond the size range of molecules to which the glomerular membrane is permeable under ordinary circumstances. One has still to explain, however, the results that  $\beta$ -lactoglobulin was cleared more rapidly in some of the experiments than any of the other proteins studied and that in general the clearance of a given protein depends upon its plasma concentration.

There is considerable evidence in the literature for the possibility of protein reabsorption in the proximal portion of the nephron (18). Monke and Yuile (8) have reported the existence of a threshold effect for the clearance of a hemoglobin by the dog. To explain this result they have assumed that approximately 2 mg. of hemoglobin per minute are reabsorbed. A mechanism for the reabsorption of protein might possibly explain the dependence of the ovalbumin and ovomucoid clearances on their plasma concentrations. If from 0.5 to 1.0 mg. of protein were reabsorbed per minute, then the application of this correction to the calculation of the clearances of these proteins would yield values independent of the serum concentration. An

absorption of this magnitude should not be sufficient to obscure the dependence of the clearance of the protein on its molecular size and shape if the results obtained at the higher plasma levels are compared.

The variation of lysozyme clearance with its plasma concentration appears to be too great to be explained solely by reabsorption. The *in vitro* experiments demonstrated the binding of lysozyme by the serum proteins of the dog. If, as the data of table 3 seem to indicate, the fraction of protein bound increases with decreasing total concentration of protein, one has at least a partial explanation for the phenomenon under discussion. Interaction of the proteins under investigation with the dog serum proteins may be a complication in every case, but the extent of the interaction is undoubtedly much greater for lysozyme than for the other proteins which were used. Electrophoretic experiments have demonstrated that lysozyme forms complexes with bovine serum albumin (13).

The only explanation which occurs to us for the anomalous results with  $\beta$ -lactoglobulin is again the formation of a complex with the serum proteins. This complex must form slowly if it is to account for the decrease in the protein clearance with

TABLE 3. EFFECT OF DOG SERUM ON THE ULTRAFILTRATION OF LYSOZYME

INITIAL LYSOZYME CONCENTRATION	LYSOZYME IN THE ULTRAFILTRATE	
	Without Serum	With Serum
mg. %	mg. %	mg. %
6.1	2.1	0.5
12.2	5.3	1.6
30.5	14.3	5.0

time at both constant and increasing plasma levels of protein. Since artificial membranes are not available which are permeable to  $\beta$ -lactoglobulin but not to serum albumin, one cannot study the *in vitro* effect of the serum proteins on the filtration of this protein as was done with lysozyme. It is probable that such an effect occurs because  $\beta$ -lactoglobulin has what Klotz (19) has termed a high 'binding index' for anions.

On the basis of the data on the physical properties of  $\beta$ -lactoglobulin one would not have expected it to be cleared as much more rapidly than ovalbumin as it was in some of the experiments. Bott and Richards (5) have also found that  $\beta$ -lactoglobulin is cleared more rapidly than ovalbumin by the frog kidney. The percentages of both ovalbumin and  $\beta$ -lactoglobulin which were cleared in their experiments are considerably higher than those obtained in the experiments reported here. Differences in the experimental techniques employed and the fact that these workers used protein concentrations from one to five times greater than were used in the present experiments do not permit any conclusion concerning the relative permeabilities of the glomerular membranes of the frog and the dog.

Since conalbumin did not appear in the urine, it is surprising that a squash seed globulin preparation with a molecular weight probably in excess of 300,000 has been reported to be eliminated in the urine (20). Bayliss *et al.* (6) in a study of the

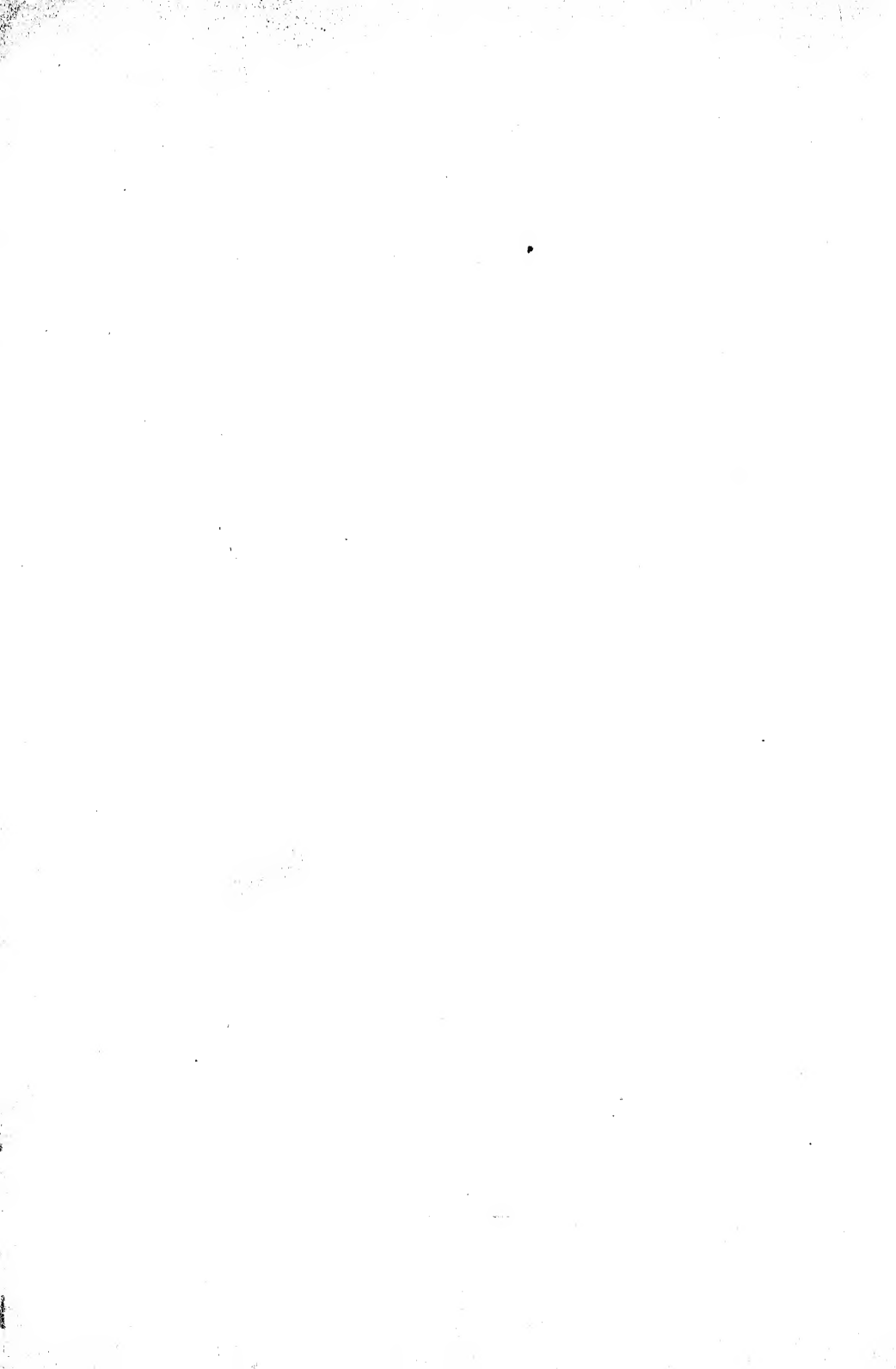
urinary excretion of 10 proteins of widely divergent size found that only those having molecular weights below 70,000 passed the glomerulus. Intravenous administration of squash seed globulin (20) or of ovalbumin (13) to the dog was usually followed by the appearance of serum albumin or globulin in the urine. In our clearance studies the total urinary protein agreed closely with that determined immunologically. This precludes the loss of any significant amount of serum protein into the urine under the conditions used in these experiments.

#### SUMMARY

The clearance by the dog kidney of 5 proteins of varying molecular size has been studied. Conalbumin with a molecular weight of 87,000 did not appear in the urine. Ovomuroid and ovalbumin were cleared to the same extent in spite of the fact that ovomuroid has a considerably lower molecular weight. This may be due to the greater asymmetry of the ovomuroid molecule. Lysozyme, the smallest protein molecule studied, was cleared more rapidly than the other egg white proteins. Anomalous results were obtained for the clearance of  $\beta$ -lactoglobulin. Its dependence on the serum concentration was much greater than were the clearances of the other proteins. The dependence of the clearances of the proteins on their serum concentrations suggests that tubular reabsorption of protein or complex formation with the dog serum proteins or both play a part in the elimination of the proteins. Definite evidence for the formation of a complex between lysozyme and the dog serum proteins was obtained. Such factors tend to obscure the dependence of protein clearance on the molecular size and shape of the molecule in question.

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## EXCITABILITY CYCLE OF MAMMALIAN AURICLE

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**R**EINVESTIGATION of the cyclical variations in excitability known to occur in the heart has been undertaken by use of methods recently described in some detail (1). It has been found that the mammalian ventricle shows oscillations of excitability during the recovery period following impulse transmission (2). The results to be reported here were obtained in a corresponding study of the dog's auricle.

### METHODS

In 35 experiments the auricles were exposed and chlorided silver driving and testing electrodes attached to the base of the right atrium near the sino-auricular node. Three electrodes were generally employed, the center one serving as a common anode for driving and testing stimuli. The sinus node was crushed to permit easier dominance by driving stimuli and slower rates of beat. Pairs of recording electrodes were attached to the central portions and apices of the right and left auricles by means of superficial stitches (3).

Responses of the auricle to driving and testing stimuli were determined by means of electrogram recording on a 12-inch cathode ray tube and by means of direct and indirect leads to a Sanborn Polyviso Cardiette.

Sodium pentobarbital (Nembutal), 30 mg/kg., was the anesthetic employed. Supplementary doses were administered as required throughout the duration of the experiments. Arterial blood pressure was recorded and the chest and body temperature maintained within a degree or two of normal levels.

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## RESULTS

The threshold excitability of the auricle during diastole remained remarkably uniform for as long as 12 to 15 hours in many cases despite the very abnormal conditions imposed by the experiment, rheobasic values frequently not changing by more than a few hundredths of a milliamperere. Variations from animal to animal were likewise surprisingly small. Figure 1 gives a comparison of auricular and ventricular late diastolic strength-duration curves obtained from the same animal. The threshold of the auricle for stimuli of short duration was generally lower than that of the ventricle. With testing stimuli of longer duration, this difference was neither conspicuous nor sufficiently frequent to warrant generalization, the auricle in a number of dogs displaying higher thresholds than the ventricle with long duration test shocks. These observations held true when the excitabilities were determined either with

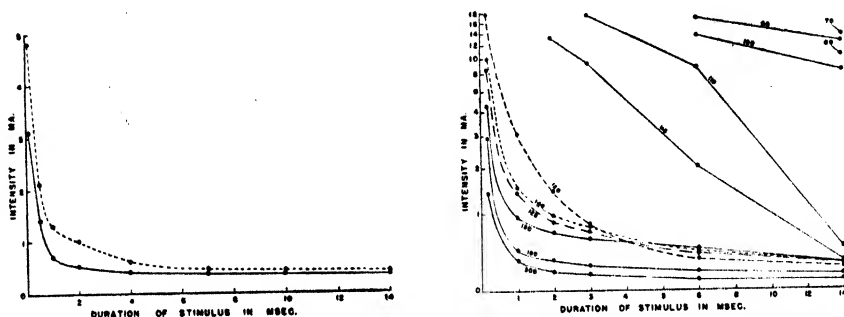


Fig. 1 (left). DIASTOLIC STRENGTH-DURATION CURVE of auricle *solid line* and ventricle *dotted line* taken within a period of 15 minutes in a representative dog. Note effect of stimulus duration on delineating differences in excitability.

Fig. 2 (right). STRENGTH-DURATION CURVES taken at various msec. intervals after origin of the beat (*exper. 116*).

bipolar or monopolar (stigmatic) techniques as well as when the heart was driven either artificially with condenser discharges or by its intrinsic mechanism.

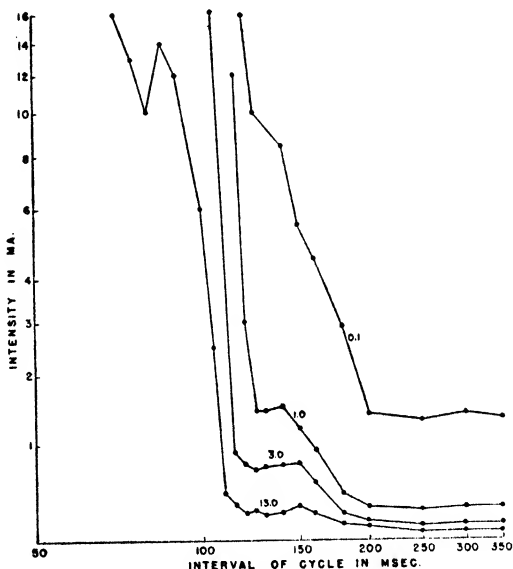
As in the case of the ventricle (2) strength-duration curves clearly revealed the different states of excitability of the heart at various intervals of the cycle (fig. 2). High intensity curves were obtained throughout the relative refractory period. The jump from the low to high intensity grouping which is seen in curves taken during terminal portions of this period reveals a difference in effectiveness of long and short duration test shocks. At those intervals during which short duration stimuli were still effective but the rheobase was significantly elevated (160-msec. curve, fig. 1) chronaxie determinations would have erroneously indicated an increased excitability.

The fact that strength-duration curves taken at certain intervals (125 and 80, fig. 2) in the relative refractory period show a greater excitability than maintains slightly later in the cycle indicates that the 'dip phenomena' or oscillations of the recovery process occur in the auricle as well as the ventricle (2). The crossing of the curves shows that stimulus shocks of different durations do not give identical definitions of the limits of the dip intervals.

Strength-interval curves (fig. 3), which were obtained by determining the strengths required by stimuli of fixed duration to stimulate at the various intervals of the cycle, gave the location of the dips and the boundaries of the refractory periods more precisely than did the strength-duration curves. As many as 3 oscillations were occasionally observed but usually only 2 were clearly present. A sharp, short, high intensity dip was revealed by long duration test shocks early in the cycle. Very rarely a secondary minor dip was seen but a relatively long-lasting major dip or flattening almost invariably occurred in the terminal portion of the refractory period.

These dips were much shallower, briefer in duration and less conspicuous than in the ventricle. They occupied approximately the same position in and the same

Fig. 3. STRENGTH-INTERVAL CURVES for 13.0, 3.0, 1.0 and 0.1 msec. duration stimuli showing dips and the boundaries of the refractory periods in one experiment (*exper. 116*).



proportion of the refractory period but since auricular refractoriness is shorter than ventricular these events were necessarily of briefer duration. The shorter auricular systolic period and the different properties of its muscular wall might also account for the smaller amplitude of the oscillations in recovery thresholds.

A comparison was made of the strength-interval curves of bipolar stimulation with curves resulting from monopolar stimulation. Results obtained with a stigmatic cathode and anode indicated, as in the case of the ventricle (2), a shift of stimulation from a cathodal to an anodal surround at certain intervals of the cycle.

The lengths of the total refractory period as determined by stimuli of various durations were similar. The durations of absolute and relative refractory periods, however, differed. Little or no absolute refractoriness was revealed by stimuli of long duration and as in the case of the ventricle it was obvious that the boundaries of absolute refractoriness were determined by the abilities of stimuli to initiate long



lasting excitatory processes (2). There was an irresponsive period in terms of the initiation of propagated impulses (4, 5) and a relative refractory period in terms of thresholds, but the auricles like the ventricle (2) were absolutely refractory to only certain durations and intensities of stimuli. According to Moe, Harris and Wiggers (6) strong shocks produce a 'polarization' of cardiac tissue which has a long-lasting effect and may result in multiple extrasystoles. Other work with various types of

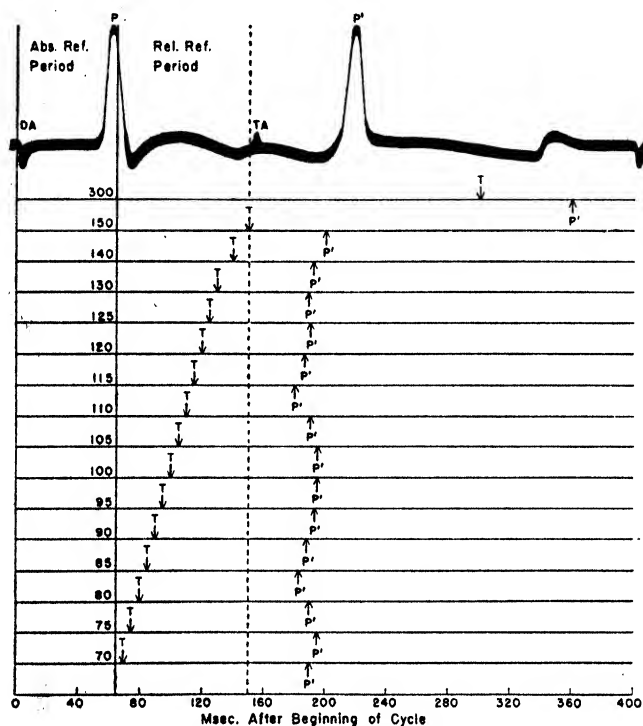


Fig. 4. RELATIONSHIP OF REFRACTORY AND IRRESPONSIVE PERIODS of the auricle to the electrogram. Increase in latency between a 0.1-msec. testing shock ( $T$ ) and induced auricular extrasystole ( $P'$ ) which occur as the stimulus is introduced earlier and earlier in the cycle is demonstrated. Auricular electrogram shows:  $DA$  and  $TA$  the driving and testing artefacts;  $P$  and  $P'$  the electrical responses to drive and test stimuli respectively.

tissue has given evidence of persistent excitatory processes created by single pulses which can outlive total refractoriness (2, 7, 8). These results indicate that an excitatory process can be initiated at any interval of the heart's cycle either within the auricular or ventricular muscle.

The exact limits of the absolute refractory period of the auricle were not easily determined, for even short duration stimuli above a certain strength give no response at all, a 'nothing-phenomenon' (3), throughout a considerable proportion of the relative refractory period. Very early in the cycle thresholds for extra systole and this nothing-phenomenon are quite close together and small increments of testing

shock must be employed to avoid jumping from a subthreshold intensity to a strength above that which will produce an extrasystole. This phenomenon has been discussed in papers dealing with auricular fibrillation which also is initiated by suprathreshold stimuli at certain intervals of the cycle (3).

Figure 4 portrays the way in which latencies of response change as the testing shocks are applied progressively earlier in the cycle. During diastole when excitability is normal the latency between stimulus artefact and the propagated response is constant and varies little for different pulse durations (0.1–15.0 msec.). As the stimuli enter the refractory period in their progressive advancement the response lags farther and farther behind as though it encounters a barrier beyond which it can not occur. Latencies, consequently, become progressively longer and the ability of a stimulus to produce a response depends upon the persistence of the excitatory state produced. The dips in the excitability curves merely represent intervals at which long-lasting excitatory processes can be created at relatively lower intensities of stimulation.

Short duration stimuli applied to the ventricle produce longer-lasting excitatory states (latencies) in terms of milliseconds than can the same stimuli when applied to the auricle. The thinner auricular wall may provide less opportunity for survival but it seems more reasonable to assume, until there is evidence to the contrary, that the shorter auricular latencies are due to the briefer refractory period and the steeper recovery gradient.

Slight changes in threshold suggestive of a post-refractory supernormality were more frequently seen in the auricles than in the ventricles. These periods of reduced threshold were found in 10 (29%) of the 35 experiments under discussion. This supernormal period was characteristically long in duration (50–100 msec.) and shallow showing a reduction in threshold of 0.05 to 0.07 ma. or 25 to 30 per cent. It resembled a terminal dip of a dampened oscillation. As in the ventricle, the early high intensity dips were sharp, close together and of short duration; the major dip though more conspicuous because of its longer duration was frequently shallower. The fact that thresholds were often higher at the 300- and 350-msec. interval in a 400-msec. cycle than they were slightly earlier might be interpreted as indicative of late subnormality rather than early diastolic supernormality. None of the 'supernormalities' recorded appeared to be typical of those described in the literature. The changes resembled the terminal phases of the oscillatory phenomenon which characterize the recovery process. The more frequent appearance of this post-refractory oscillatory dip in the auricular than in the ventricular excitability curve might be accounted for by the steeper recovery gradient in the former tissue.

The differences between the phenomena of auricular and ventricular excitability and excitation were ones of degree rather than kind. The effects of drugs, rate of beat and other influences on excitability and conduction in the auricle will be dealt with in subsequent communications.

#### SUMMARY

A comparison was made of the auricular and ventricular excitability cycle. The phenomena of excitation were essentially the same in the two tissues. The

auricle has a slightly lower threshold than does the ventricle under the conditions described. Recovery of excitability following a beat is not a smoothly progressive process but reveals oscillatory changes which create dips or periods of relative supernormality during the cycle.

All phenomena of the refractory period, latencies, dips, etc., are shorter in duration and less conspicuous than in the ventricle. A post-refractory period of slight supernormality was observed more frequently in the auricle than in the ventricle. As in the ventricle the length of the absolute refractory period depends upon the duration and strength of the stimulating pulse.

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# POSITIONAL CHANGES OF THE HEART AND THEIR EFFECTS ON ELECTROKYMOGRAPHIC RECORDINGS<sup>1</sup>

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**D**URING ejection of blood the walls of the heart move inward and during filling they move outward. These changes in thickness of the heart can be recorded using the electrokymograph (EKY) as previously described (1). The accurate measurements of these inward and outward movements would be a simple matter if positional movements of the heart did not distort the records. It is the purpose of this paper to discuss these positional changes, the manner in which they affect the EKY records and the ways of circumventing this difficulty so as to record true inward movements of the cardiac muscle walls.

It must be remembered that the EKY record changes in x-ray absorption. We have used it with the sensitive portion of the head completely covered by heart shadow. The records obtained are called densograms. A downward deflection of the galvanometer string indicates decreased x-ray absorption and may occur as a result of inward movements of the cardiac muscle or because a thinner portion of the heart moves under the EKY head. It is obvious that one may get records in which the change in position of the heart adds to or subtracts from the change in x-ray absorption caused by contraction of the cardiac muscle.

## METHOD

Dogs anesthetized with sodium pentothal and barbital were placed in the left lateral position and densograms were systematically recorded over every portion of the heart shadow. A lead plate with an opening one cm. square was placed in front of fluorescent screen of the EKY head. Then the head, centered over the x-ray beam, was moved with the x-ray tube to a portion of the heart's shadow close to the caudal left or right edge of the heart. At least 20 records of beats were obtained. During alternate beats, pressdwood, 3.1 mm. thick, was automatically moved into and out of the x-ray beam. The response to the pressdwood provided a calibration which made it possible to compare amplitude of the responses from different areas (1). Simultaneously with the EKY records, pressure pulses from the root of a carotid artery were recorded with a strain gauge. The moveable system carrying the EKY head and x-ray tube was then moved one cm. laterally or cephalad over the heart shadow and the observations were repeated. This was continued until records were obtained

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over the entire silhouette. Using the calibration responses, the records were calculated in terms of actual thinning or thickening of the heart ( $x$ ) and plotted in the appropriate area of the heart shadow. Such heart maps of EKY responses were drawn for dogs and subsequently for human subjects.

The second part of this work consisted in recording the movements of the dog's heart. In dogs under sodium pentothal and barbital, the 4th or 5th rib on the left side was removed and the chest was opened under artificial respiration. A J incision was made in the pericardial sac and stainless steel markers with barbed points were pushed into the ventricles on both the upper and under surfaces. The pericardium and chest wall were then closed. Air was removed from the pleural space and normal pressure relations were re-established. Then with the animal breathing slowly, the movements of the markers were recorded. This was done in two ways. The first and less accurate method consisted of obtaining x-ray pictures of the heart automatically exposed at various time intervals after the R wave of the ECG. Simultaneous records of the carotid pressure pulse and time of x-ray exposure showed the moment in the cardiac cycle at which the picture was taken. By comparing a number of these plates the movements of the stainless steel points could be determined.

Another method for determining movements of the points utilized the EKY. A lead plate with a 3-mm. square opening was placed on the EKY head so as to block all off x-rays except those passing through this small opening. The movable system carrying the EKY head and the x-ray tube was then moved so that the head was centered just outside the path of a stainless steel marker. At least 10 simultaneous carotid pressure pulses and EKY densograms were recorded, care being taken that the observations were made while the diaphragm was in the expiratory position. The EKY head and x-ray tube were then moved 2 mm. laterally toward the path of the marker and 10 beats were again recorded. If the steel marker moved under the EKY head, a spike was produced and provided a means of measuring the moment in the heart cycle that the marker reached that position. These recordings were continued, moving laterally in steps of 2 mm., until the EKY head was beyond the area where spikes occurred. Then the head and tube were moved 2 mm. cephalad or caudad and after recordings were obtained, the EKY head and x-ray tube were moved laterally again in steps of 2 mm. so as to pass through the path of the marker. In this manner the entire area into which the marker moved was systematically surveyed.

With graph paper, the positions of the EKY tube were carefully marked out and time in the cardiac cycle when a spike was observed was indicated in the appropriate square. A line connecting the times in proper sequence indicated the path of the marker.

As this work progressed, it was noted that the position of the heart in the chest was affected by changes in blood pressure. In order to understand how this might modify the EKY records it was important to know the direction and magnitude of these movements. Then one should be able to predict not only how a change in arterial pressure but also changes in pulse pressure might modify the records. For this study, we placed x-ray opaque markers on the heart and the great vessels. The x-ray plates were then taken during the same phase of the cardiac cycle but with elevated or reduced blood pressure. The R wave of the ECG plus an appropriate lag

was used to time these x-ray exposures. The blood pressure was varied by giving either epinephrine or sodium nitrite. A graphic record of arterial pressure and the time of the x-ray exposure was obtained so that if any errors in timing occurred, they were at once apparent and the plates incorrectly exposed could be omitted.

### RESULTS

In figures 1 and 2, the average EKY records for a complete cycle are shown in appropriate places on the silhouette. These views are of the dog in the left lateral position. The right ventricle covers the sternal area (left side of fig.) and the left

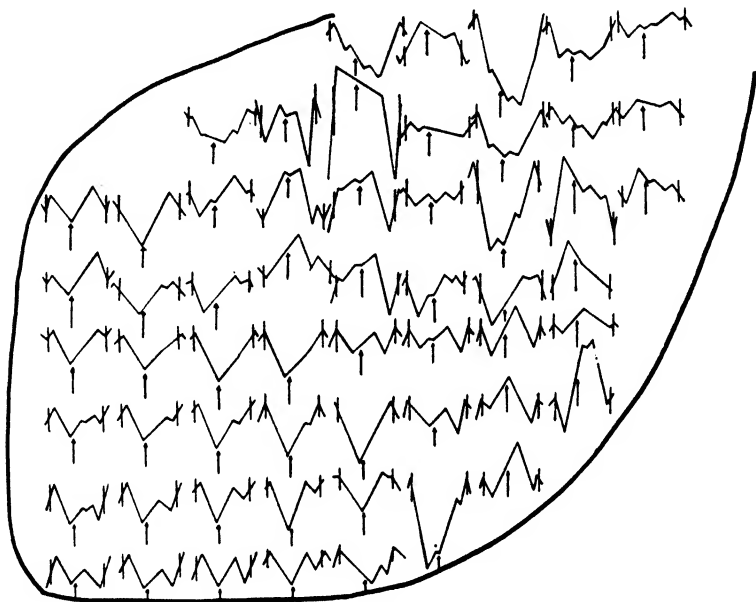


Fig. 1. WITH DOG in left lateral position, EKY densograms for each  $\text{cm}^2$  of heart silhouette are shown. Sternal side is on left and spinal on right. Much of upper caudal surface of heart in this view is left ventricle. Vertical lines mark beginning of ejection. Complete cycle is shown between 2 vertical lines. Arrow head indicates end of ejection.

ventricle the caudo-spinal area. The vertical lines in each case mark the beginning of ejection as determined by the carotid pulse. A complete cycle is therefore shown between 2 vertical lines. The arrow heads indicate the end of ejection. Downward movement represents an inward movement or decrease in absorption of x-rays. On these graphs, no correction has been made for time required for the pulse to reach the recording strain gauge.

One point of interest is the timing of peaks which appear during systole. On the sternal side of the heart, it will be noted that these peaks occur some time after ejection starts. But as one moves over toward the spinal side, the peaks occur earlier in ejection and finally precede ejection. This is especially well shown in the third row

up from the apex. In this row, the inward movement starts with beginning of ejection in the space 4 cm. over from the sternum. If one assumes that the isometric contraction causes the changes indicated in figure 3, then the apparent movement during this period would be outward on the sternal side and inward on the spinal side. If

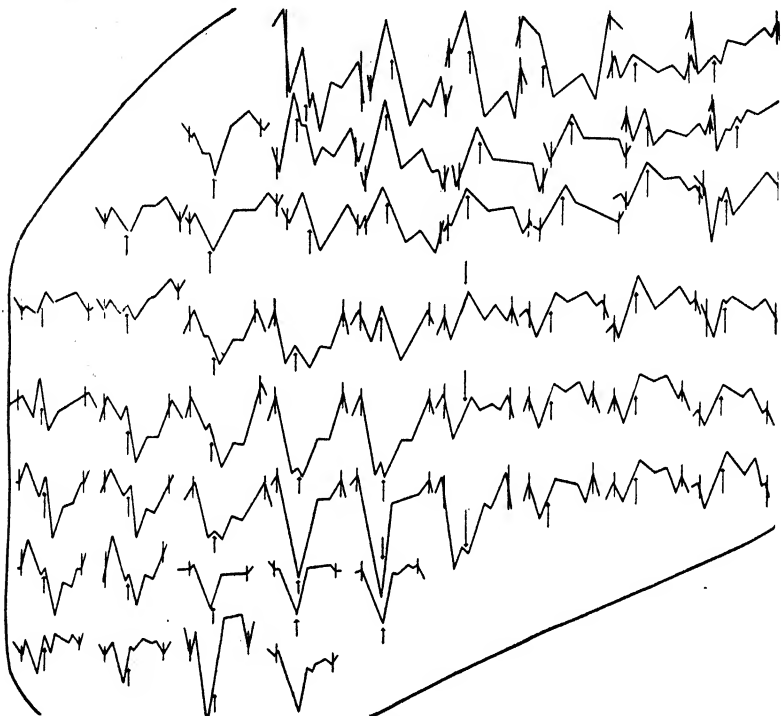


Fig. 2. SEE legend for figure 1.

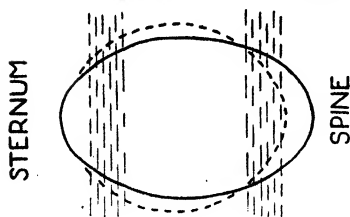


Fig. 3. CHANGES IN CROSS SECTION of heart believed to occur during isometric contraction.

one further assumes that the heart moves sternally during ejection (and this will be shown to be true), then an outward movement on the sternal side and inward movement on the spinal side during early ejection can be explained.

The movement of the base of the heart toward the apex during ejection may also distort these records because, in this case, a thicker area of the heart may move under the EKY head. This is especially noticeable in some records from the spinal side of the heart. Here one frequently observes an apparent thickening during ejection and thinning during filling.

One should also note especially that in the third row above the apex the amplitude of the responses increases as one moves from the sternal to the spinal side of the heart. This can be explained by the fact that the positional shift cuts off a part of the inward movement on the sternal side and adds to the true inward movement on the spinal side.

The responses shown in figure 1 are simpler and less distorted than those in figure 2. In the latter figure, one notes on the sternal side an outward movement at the beginning of systole and again toward the end. An inward movement at the beginning of diastole still further distorts the records. It appears that the movement toward the sternum during ejection modifies the end as well as the beginning of the

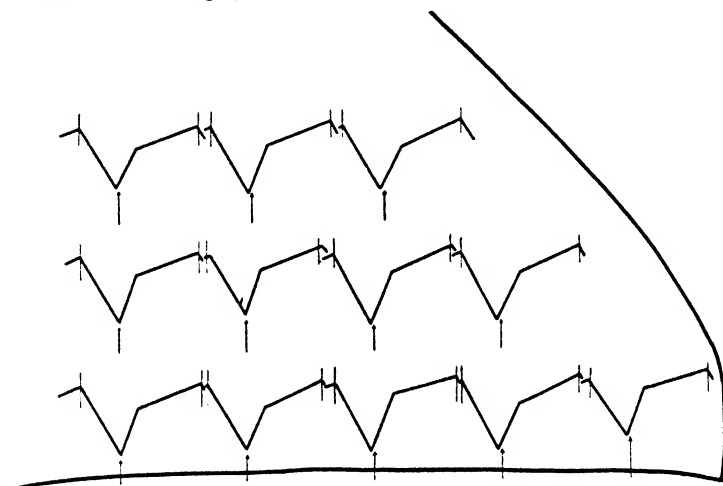


Fig. 4. EKY DENSOGRAMS for each cm.<sup>2</sup> of a human-heart silhouette in antero-posterior view. Diaphragm is shown below; spinal column on left.

ejection period and that a movement away from the sternum is probably responsible for the apparent inward movement during early diastole.

One can note in the atrial areas inward movements which start about the time that atrial contraction begins. Since positional changes cause such marked effects, one would hesitate to use these areas for timing or for amplitude measurements. In both figures 1 and 2, one can observe in the upper right area, records which have somewhat the appearance of arterial pulses though in each case some distortion is obvious. This is where one would expect to record pulmonary and aortic pulses.

It should be emphasized that we have considered only the effects of caudo-sternal displacement of the heart. The heart also rotates during contraction. The distortions caused by this will be considered in another paper.

The human heart exhibits much smaller positional changes than does that of the dog, no doubt because of the less elastic mediastinum. Therefore the EKY density pattern over wide areas is reasonably constant.

For comparison with the dog's EKY records taken in the lateral position, we have used human records taken in the AP position. The dog's heart is thinner in the lateral than in the AP dimension whereas the reverse is true of the human heart.



The records of human heart in AP position are quite similar to those of the dog in the lateral position. Figure 4 shows such records calculated in terms of calibration responses. They show little change in the amplitudes or contour. Occasionally one observes in a human subject a map of EKY responses of the ventricular area which is similar to that noted in figure 1 for the dog. However the shift in the timing of the systolic peak as one moves from one area to another is much smaller and the amplitude changes much less than those noted in the dog.



Fig. 5. MOVEMENTS OF MARKERS on upper surface of the dog's heart in left lateral view. Sternum is on left and spine on right. *Full lines* represent period of ejection and *broken lines* rest of the cycle. *Arrow head* between ends of *full line* is at .10 sec. after beginning of ejection. *Arrow head* between ends of *broken line* indicates beginning of isometric contraction. Most of these points were on left ventricle. A few on sternal side were on right ventricle. A and B: dog 1; C, D and E: dog 2; F, G and H: dog 3; I, J and K: dog 4; L, M and N: dog 5; O, P and Q: dog 6; 1: slow heart rate; 2: fast heart rate.

The positional movements of the dog's heart are shown in figures 5 and 6. These are left lateral views. The upper surface in this position (fig. 5) is largely left ventricle with some right ventricle on the sternal side. The under surface (fig. 6) is largely right ventricle with some left ventricle on the spinal side. Most of the upper points therefore show movements of the left ventricle and most of the under points movements of the right ventricle. The full lines represent the period of ejection and the broken lines the remainder of the heart cycle. During early ejection the movements are shown to be predominantly caudal and sternal with large movements at the base and smaller ones at the apex. Hamilton and Rompf (2) have shown that in

dogs the base of the heart moves toward the apex during ejection. Westermarck (3) has placed metallic markers on the sheep's left ventricle and using a fluoroscope has taken moving pictures of their movements after the chest was closed. He found that a point on the base of the left ventricle moved downward and to the left in isometric contraction and upward, forward, and to the right later in systole. Points half way between the base and apex moved to the left and backward in isometric contraction and later, during ejection, to the right, forward, and caudally. The apex appears to

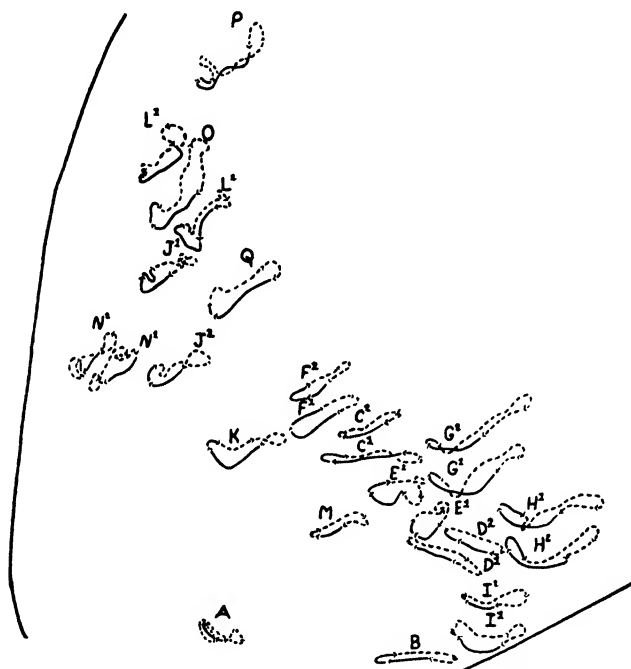


Fig. 6. SAME AS FIGURE 5 except that markers were on under surface and were mostly in the right ventricle. A few on the spinal side were on left ventricle. A: dog 1; B and C: dog 2; D, E and F: dog 3; G, H and I: dog 4; J, K, L and M: dog 5; N, O, P and Q: dog 6; 1: slow heart rate; 2: fast heart rate.

be relatively fixed. These observations on the sheep differ in timing from ours on the dog but during the major part of ejection the hearts of sheep and of most dogs show a sternal and caudal movement through the middle areas of the left ventricle.

The movements on the right side of the heart (under surface) are much greater than those on the left and in early ejection are predominantly sternal. Therefore it appears that during this period the heart rotates in a clockwise direction if observed from a caudal position. Either late in systole or early in diastole, depending probably on the length of the cycle, a movement toward the spine is very apparent on the right side and suggests that a contra-clockwise rotation is occurring. This continues up to the time of isometric contraction. Then clockwise rotation begins and lasts through

most of the ejection period. These observations differ from those of Burchell and Visscher (4) who took high-speed motion pictures of the dog's heart with the chest open. The differences between their observations and ours are probably explained by the open chest and the reduction in the support which is normally given by the lungs and the pericardium when the chest is closed. Our observations concerning rotation of the dog's heart also differ from the descriptions usually given for the human heart (5).

A caudal movement lasting for the first 0.08 to 0.10 second of the ejection phase is usually observed on both the left and the right sides of the heart. This thrust corresponds roughly to the timing of the maximal pressure observed on arterial pressure records. We have therefore wondered what effects changes in pressure *per se* have on the position of the heart. A typical example of the results obtained from recording the position of points on the heart during the same phase of the cardiac cycle but under different conditions of pressure is shown in the figure 7. The x-ray

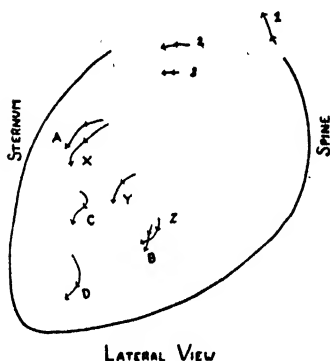


Fig. 7. EFFECTS OF RISING arterial pressure on markers placed on the heart and great vessels. 1: arch of aorta; 2: ascending aorta; 3: pulmonary artery; A, B, C and D: markers on left ventricle; X, Y and Z: markers on right ventricle; blood pressure after  $\text{NaNO}_2$ —84/52; normal pressure (arrowhead in middle of line)—150/97; after epinephrine—250/180.

plates from which this figure was drawn were made during early systole but similar changes are observed in early diastole. It appears that with rising pressures the ventricles push downward and the aorta moves upward.

#### DISCUSSION

This work was started in order to find where, on the heart silhouette, the EKY records would be least affected by positional changes. In dogs between 30 to 60 pounds, this area in the left lateral view has been found to be about 3 cm. cephalad from the apex and 4 cm. over from the sternal edge of the heart. This is satisfactory because the portion of the heart which moves under the EKY head during the ejection has the same diastolic thickness as that part of the heart which it replaces. The positional shift therefore neither adds to nor subtracts from the change in x-ray absorption due to the inward movement of the cardiac walls.

The above position is the best one yet discovered for measurement of cardiac output. With the EKY head placed over this area a satisfactory correlation between heart output measured by the Fick or Stewart principle and the EKY measurements has been obtained (6, 7).

Only the EKY responses in the left lateral view have been carefully investigated. It may be found that in an oblique view, true inward movements are even less distorted than in the lateral view. This is now being investigated.

One should point out that records of border movements of the heart and great vessels are seriously distorted by positional changes. In fact the movement cephalad of the aorta during ejection may, if the EKY head is placed over the caudal surface of this vessel, record an apparent inward motion during this period. One must therefore be very cautious in using this instrument for timing of cardiac events.

#### SUMMARY

In dogs lying in the left lateral position, a satisfactory record of the movements of the ventricles using the electrokymograph can be obtained if the head is placed in the proper position over the cardiac silhouette, namely 3 cm. cephalad from apex (see figs. 1, 2), and 4 cm. dorsal of spinal edge. When the head is moved to other positions distortions occur which can be explained by the known positional movements of the heart (see figs. 4-6). Electrocardiographic density records obtained from the human heart are usually satisfactory over wide areas (see fig. 3). This is probably due to the fact that the human heart shows smaller positional movements. With a rise in blood pressure the heart moves downward and the aorta moves upward.

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# BLOCKADE OF EPINEPHRINE-INDUCED CARDIOACCELERATION IN THE FROG<sup>1</sup>

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**M**ANY workers have reported the failure of adrenergic blocking agents to prevent stimulation of the mammalian myocardium by epinephrine (1). All the thoroughly studied groups of blocking agents are uniformly ineffectual; these include the  $\beta$ -haloalkylamines, the ergot alkaloids, the imidazolines, the benzodioxanes and yohimbine and its congeners. However, the results of experiments employing the frog heart have been highly variable. A survey of the literature on this subject reveals a number of reports which indicate that the  $\beta$ -haloalkylamines, the ergot alkaloids, the benzodioxanes and yohimbine block or reverse the stimulant actions of epinephrine. Many other investigators have reported these same agents to be as ineffective in blocking the effects of epinephrine on the frog heart as on the mammalian heart (1). The present literature on this subject provides no adequate explanation for the discrepancies.

Preliminary studies in our laboratory indicated that Dibenamine and several of its congeners, and a number of ergot alkaloids are all capable of blocking and reversing the chronotropic action of epinephrine on the isolated, perfused frog heart when employed in very low concentrations. However, subsequent efforts to extend these observations led to highly conflicting results and consequently a systematic study of the conditions necessary for blockade was undertaken.

## METHODS

All experiments were performed on the isolated, perfused hearts of *Rana pipiens*. Frogs were obtained from E. G. Steinhilber, Oshkosh, Wisconsin. These animals were freshly caught during the period of March to November and were kept in sheltered trenches at a water temperature between 32° and 40°F. during the period of late November through February. After arrival in our laboratory the animals were kept at room temperature in tanks containing about one inch of water which was changed daily. Room temperatures varied between 68° and 76°F. during the winter and up to 90°F. in the late summer. The perfusion experiments were carried out at room temperature.

In most experiments the heart was prepared according to the single cannula technic of Straub, adapted to provide a continuous flow of the perfusate. A perfusion pressure of about 5 cm. of water was employed. The perfusion solution was introduced through a fine needle which extended to the tip of the glass cannula. This provided a rapid exchange of the perfusate similar to that obtained with the cannula pictured by Krayner *et al.* (2), and largely eliminated the 'dead space' of the single cannula. A perfusion rate of 5 to 7 ml/min. was employed. Two reservoirs of perfusate were attached

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<sup>1</sup> This investigation was aided by research grants from the National Institutes of Health, Public Health Service; the Smith, Kline and French Laboratories; and Sandoz Pharmaceuticals.

to the needle leading into the cannula in such a way that the perfusion solution could be changed rapidly by switching from one to the other. In a few experiments the double cannula of Clark was used, but the results obtained by this method were not significantly different from those obtained with the simpler single-cannula preparation. Howell frog-Ringer solution (0.70% NaCl, 0.03% KCl, 0.026%  $\text{CaCl}_2$ , 0.003%  $\text{NaHCO}_3$ , pH 7.3) made with glass-distilled water was employed throughout. In some experiments, 5 per cent beef serum was added to the solution in order to provide for a longer period of uniform heart action. A series of duplicate experiments, in which the salt solution with and without added serum was employed, demonstrated no significant difference in the response of the heart to epinephrine or in the specific effects of added inhibitors or metabolites. However, the addition of serum did allow for longer periods of perfusion with higher concentrations of blocking agents than were possible with the unsupplemented frog-Ringer solution.

The response of the heart to epinephrine was determined by injecting this agent (usually 1.0  $\mu\text{g}$ .), contained in 0.5 ml. of solution, into the perfusion system just above the entrance into the cannula. In experiments designed to elucidate the quantitative aspects of the observed antagonisms, varying doses of epinephrine were injected in the same volume to assure a constant dilution factor. The amplitude of contraction of the heart was recorded by means of an end-writing lever, and the heart was allowed to work against a weight of approximately one gram. The rate was determined by counting the number of beats per 30-second period prior to each injection of epinephrine, and continuously after the administration until it returned to the control level, usually within 2 to 3 minutes. The results are recorded in terms of the minimum concentrations of the various agents required to inhibit or reverse the chronotropic response to the added epinephrine or to eliminate this blockade after it was produced (figs. 3 and 4). Changes in rate of one beat per 30-second period were considered insignificant because of error in counting. No distinction has been made between complete blockade of the chronotropic response and its 'reversal.' However, in many experiments, after blockade was produced, the heart rate decreased 2 to 6 beats per minute in response to epinephrine (figs. 3 and 4) and in occasional preparations the epinephrine-induced bradycardia amounted to 16 to 20 beats per minute. This variation in response appeared to depend more upon the characteristics of individual preparations than upon the particular blocking agent employed.

All perfusates were prepared immediately prior to use and solutions containing  $\beta$ -haloalkylamines or other unstable agents were made up fresh each half hour during prolonged experiments. Each concentration of a drug or combination of drugs was allowed to perfuse for 45 to 60 minutes unless a definite positive effect was obtained earlier.

## RESULTS

*Various Blocking Agents.* The first experiments involved a study of the blocking actions of the ergot alkaloids<sup>2</sup>—ergonovine, ergotamine, dihydroergotamine and dihydroergocornine—and Dibenamine<sup>3</sup> (N,N-dibenzyl- $\beta$ -chloroethylamine) and its congeners—G-D 52 (N-benzyl-N-ethyl- $\beta$ -chloroethylamine) and G-D 218 (N-o-methylphenoxyethyl-N-benzyl- $\beta$ -chloroethylamine). G-D 52 is significantly less potent and G-D 218 considerably more potent than Dibenamine when tested against the pressor response to epinephrine in cats (3, 4). Ergotamine and ergonovine were employed as the tartrates, dihydroergotamine and dihydroergocornine as the methanesulfonates, and the  $\beta$ -haloalkylamines as the hydrochlorides. The first experiments were performed during January 1949 in an attempt to reconcile the divergent results reported in the literature and mentioned above. Each of the adrenergic blocking agents tested was found to act in low concentration to inhibit or reverse

<sup>2</sup> The ergot alkaloids were kindly supplied by Mr. Harry Althouse, Sandoz Pharmaceuticals, San Francisco, California, and the  $\beta$ -haloalkylamines by Dr. William Gump, Givaudan-Delawanna, Inc., Delawanna, N. J. N,N-dibenzyl- $\beta$ -chloroethylamine is now distributed for investigational use by the Smith, Kline and French Laboratories, Philadelphia 1, Pa., under their trademark Dibenamine.

the chronotropic response to epinephrine, but none of them prevented the positive inotropic action when employed in concentrations which did not depress the spontaneous activity of the heart. This dissociation of the chronotropic and inotropic responses is illustrated in the records shown in figure 1. The minimum effective concentrations of these agents are shown in table 1, and are quite comparable to those required to produce blockade of epinephrine stimulation of smooth muscle structures. The required concentrations are sufficiently low to indicate that a relatively specific blockade is involved. G-D 12 (2-dibenzylaminoethanol, the adrenergically inactive hydrolysis product of Dibenamine), G-D 51 (N,N-diethyl- $\beta$ -chloro-

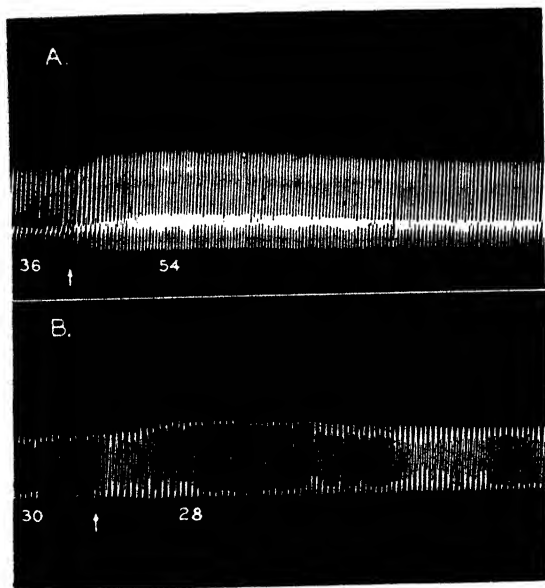


Fig. 1. KYMOGRAPH RECORDS illustrating the response of the isolated frog heart to 1.0  $\mu$ g. of epinephrine. A. Control. B. After blockade with 0.5  $\mu$ g./ml. of G-D 218. Arrows indicate time of epinephrine administration and figures the heart rate in beats per minute at the designated point. Time in 30-second intervals.

ethylamine, an adrenergically inactive saturated congener) and ergonovine are ineffective.

Chronotropic responses to norepinephrine and isopropylnorepinephrine are blocked as readily as those to epinephrine. Doses of 0.5  $\mu$ g. of L-isopropylnorepinephrine<sup>3</sup> and 10  $\mu$ g. of DL-norepinephrine<sup>3</sup> were found to produce essentially the same chronotropic response as 1.0  $\mu$ g. of L-epinephrine, and to be blocked by the same concentrations of G-D 218 and dihydroergocornine.

The blocking action of Dibenamine and its congeners on the frog heart differs in one important respect from their action on mammalian smooth muscle (1, 5). The blockade does not persist for any considerable period of time after the perfusion containing the active agent is replaced by fresh frog-Ringer solution. This difference represents a special problem which is currently being investigated.

<sup>3</sup> Isopropylnorepinephrine was kindly supplied by Dr. E. L. Burbridge, Frederick Stearns & Co., and norepinephrine by Dr. M. L. Tainter, Sterling-Winthrop Research Institute.

*Seasonal Variations.* During February and March 1949 an attempt was made to extend these observations to other congeners of Dibenamine, but the results were highly variable. By April it was found that even G-D 218, which had previously been the most potent blocking agent, was completely ineffective even when perfused in the maximum doses tolerated by the excised heart. At this time dihydroergocornine still produced a blockade, but concentrations nearly 10 times as great as those initially employed were required.

These results indicated a seasonal variation in the sensitivity of the frog heart to adrenergic blockade and consequently the minimum dose required to produce blockade was determined at intervals, at least twice a month, during the remainder of the year (fig. 2). G-D 218 and dihydroergocornine were selected for these experiments because they were the most potent of the agents studied.

Seasonal variations in sensitivity to the G-D 218 blockade were most marked. Although the chronotropic response to epinephrine was completely blocked by 0.2

TABLE 1. BLOCKADE OF THE CHRONOTROPIC RESPONSE OF THE WINTER-FROG HEART TO EPINEPHRINE BY VARIOUS AGENTS

TIME OF YEAR	BLOCKING AGENT	MIN. BLOCKING CONC.	TIME OF YEAR	BLOCKING AGENT	MIN. BLOCKING CONC.
		$\mu\text{g/ml.}$			$\mu\text{g/ml.}$
Jan.	Dibenamine	1.0	Jan.	Ergotamine	1.0
Jan.	G-D 52	2.0	Jan.	Dihydroergotamine	1.0
Jan.	G-D 218	0.2	Jan.	Dihydroergocornine	0.5
Jan.	G-D 12	> 15.0	Jan.	Ergonovine	> 15.0
Jan.	G-D 51	> 15.0			

$\mu\text{g/ml.}$  during the period of maximum sensitivity, concentrations of 7.5 to 10.0  $\mu\text{g/ml.}$  were completely ineffective between April 1st and August 1st. The change in sensitivity during August occurred within a single shipment of frogs while the animals were kept under relatively constant conditions in our laboratory. The seasonal variation in sensitivity to dihydroergocornine is somewhat less marked, but it is well correlated chronologically with the alteration in sensitivity to G-D 218. The hump in the dihydroergocornine curve during April and May appears to represent a real effect which has been confirmed during a second season.

The observed variations in sensitivity to blockade are not well correlated with environmental temperature changes. A reduction in sensitivity occurred before marked changes in temperature in the spring of the year, and the sensitivity returned to a major extent during the very warm month of August. In addition all the frogs studied had been kept in indoor laboratory tanks for one to 6 weeks prior to use.

However, low temperatures were found to produce significant changes in sensitivity when the frogs were exposed to the altered temperature for an extended period. When the animals were kept in a refrigerator at 8°C. for 7 to 15 days, in both July and October, a significant reduction in the minimum effective dose of G-D 218 or dihydroergocornine occurred (table 2). Cooling the animals for 24 to 72 hours was



ineffective. Unfortunately, no studies of the effects of reduced environmental temperature were undertaken during the early part of the resistant period. Cooling the perfusate to 12°C. markedly reduced the heart rate and the response to epinephrine even in the absence of a blocking agent. However, within 5 to 10 minutes after the solution was again warmed to room temperature resistance to blockade was the same as before cooling. This was noted even after the heart had been exposed to the cooled perfusion solution for as long as 5 hours.

*Anterior Pituitary Effects.* The sensitivity of the frog heart to blockade of the chronotropic response to epinephrine thus varies with the season, but is not well correlated with the seasonal temperature variations. In addition, prolonged changes

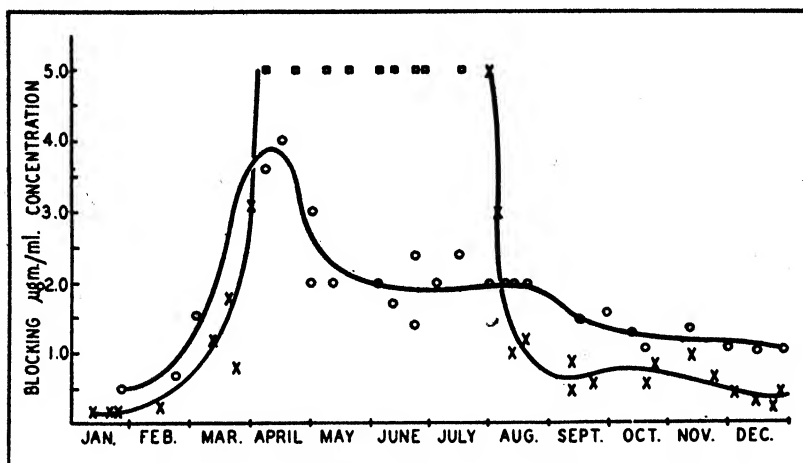


Fig. 2. SEASONAL VARIATION in sensitivity of the isolated frog heart to blockade of the chronotropic response to epinephrine. X marks indicate minimum blocking concentrations of G-D 218; squares indicate tests with G-D 218 which produced no blockade; circles indicate minimum blocking concentrations of dihydroergocornine.

in environmental temperature within the laboratory may alter sensitivity although short-range changes do not. These observations indicate that the effects of changing seasons and temperatures are mediated through some body system which requires a considerable period of time for activation or inhibition and which may also be affected by factors other than temperature. In addition, the decrease in sensitivity observed during February and March appears to be well correlated chronologically with changes in gonadal function which are known to be controlled by the anterior pituitary gland. Consequently, the effect of injected pituitary extract upon the sensitivity of the winter-frog heart to adrenergic blockade was investigated.

Lyophilized hog whole or anterior pituitary tissue<sup>4</sup> (10 mg/ml.) was extracted with frog-Ringer solution. Even large amounts (1.0 ml/frog) of this extract injected into the ventral lymph sac of winter frogs kept at 8°C. failed to alter the sensitivity of the hearts to blockade during periods up to 21 days. However, small amounts

<sup>4</sup> Kindly provided by Dr. George Sayers, University of Utah College of Medicine.

injected into animals kept at room temperature produced, within 24 hours, a complete resistance to blockade by G-D 218 and markedly increased resistance to dihydroergocornine (table 3). The hearts of winter frogs treated in this manner were found to be indistinguishable from summer-frog hearts in their response to adrenergic blocking agents. The addition of pituitary extract to the perfusion solution produced no changes in sensitivity even with concentrations up to 4 per cent and perfusion times as long as 5 hours.

Anterior- and whole-pituitary extracts produced the same effects in these experiments, and consequently it may be assumed that some principle extracted from the anterior pituitary tissue is responsible for the observed alteration from the winter- to the summer-type response. Uninjected winter frogs kept at room temperature were quite sensitive to blockade during the period of these experiments.

TABLE 2. EFFECTS OF SEASON AND TEMPERATURE ON THE BLOCKADE PRODUCED BY G-D 218, DIHYDROERGOCORNINE AND G-D 218 PLUS FLUOROACETATE

TIME OF YEAR	TREATMENT OF FROGS	AV. MIN. BLOCKING CONC.		
		G-D 218	Dihydroergocornine	G-D 218 + $3 \times 10^{-4}$ Fluoroacetate
		$\mu\text{g/ml.}$	$\mu\text{g/ml.}$	$\mu\text{g/ml.}$
Jan.-Feb.	Control Room Temp.	0.2	0.6	0.2
June-July	Control Room Temp.	> 10.0	2.2	0.2-0.5
Sept.-Nov.	Control Room Temp.	1.0	.3	0.1-0.2
July	9 days at 4°C.	0.2		0.2
Oct.	12 days at 8°C.	0.2	0.5	0.2
Oct.	48 hr. at 8°C.	1.0		
Oct.	Perfused 5 hr. at 12°C.	1.0		
Jan.	Ant.-pit. extr.; 24 hr. at room temp.	> 10.0	3.0	0.2

It is probable that the failure of animals kept at 8°C. to respond to the pituitary injection is due to a lack of utilization of the active principle at this temperature.

*Combinations of Inhibitors.* Both the inotropic and chronotropic responses of the heart to epinephrine are expressions of an increased production and/or utilization of energy. In many situations where an overall metabolic process is resistant to an inhibitor known to block some stage of the process, biochemical studies have demonstrated that failure of the blockade to be manifest is due to the presence of an alternate system capable of by-passing the blocked reaction (e.g. flavoproteins and cyanide-resistant respiration). It was therefore assumed that epinephrine releases energy for the production of a tachycardia by only one mechanism in the winter-frog heart, which is inhibited by adrenergic blocking agents, whereas in the summer-frog heart two or more pathways are available for release of the necessary energy.

On the basis of these considerations, an attempt was made to block the chronotropic response of the summer-frog heart to epinephrine by combining G-D 218 or dihydroergocornine with various known metabolic inhibitors. This study was largely carried out with G-D 218 because the greater seasonal variation in the response

to this agent and other known features of its pharmacology indicate a higher specificity of action than characterizes the ergot alkaloids. Dihydroergocornine was studied with several of the inhibitors and in all cases the responses were qualitatively the same as with G-D 218.

In studies on the summer-frog heart, G-D 218 in a concentration of 5  $\mu\text{g}/\text{ml}$ . was added to the perfusing solution in addition to the inhibitor to be tested. In this concentration G-D 218 alone had absolutely no effect on the chronotropic response to epinephrine (see fig. 3 and table 4). A few additional experiments were carried out from September through November. In these experiments a concentration of G-D 218, half that required to produce blockade, was employed. Other inhibitors were added to the perfusing solution in a concentration of  $1 \times 10^{-5}$  molar, and if no activity was detected at this level, the concentration was increased, usually until

TABLE 3. EFFECTS OF PITUITARY EXTRACTS ON BLOCKADE PRODUCED BY G-D 218 AND DIHYDROERGOCORNINE

TIME OF YEAR	TREATMENT	AV. MIN. BLOCKING CONC.	
		G-D 218	Dihydroergocornine
		$\mu\text{g}/\text{ml}$ .	$\mu\text{g}/\text{ml}$ .
Oct.-Nov.	Controls, room temp.	1.0	1.3*
Oct.-Nov.	Controls, 12 days at 8°C.	0.2	0.5
Oct.-Nov.	Whole-pit. extr. 1 to 21 days at 8°C.	0.2-0.5	
Oct.-Nov.	Whole-pit. extr. 24 hr. at room temp.	>10.0	3.0
Oct.-Nov.	Ant.-pit. extr. 24 hr. at room temp.	>10.0	
Oct.-Nov.	Ant.-pit. extr. perfused for 5 hr.	1.0	

the maximum concentration tolerated by the heart had been reached or activity was obtained.

The results of these experiments are summarized in table 4. There is a sharp distinction between those metabolic inhibitors which produce a blockade of the chronotropic response of the summer-frog heart to epinephrine when combined with G-D 218 and those which do not. Of the group studied only monofluoroacetate and monoiodoacetate are effective, and these agents act in very low concentrations (see also fig. 3). Chronotropic responses to norepinephrine and isopropyl norepinephrine were inhibited in exactly the same way as those to epinephrine. None of the metabolic inhibitors blocked the chronotropic response when tested alone, even in the maximal tolerated concentration, and none of the agents alone or combined with G-D 218 or dihydroergocornine prevented the inotropic response to epinephrine under the conditions of our experiments. The various blocking combinations were tested both in the presence and in the absence of serum in the perfusion solution. The blockades produced with fluoroacetate and iodoacetate were qualitatively and quantitatively the same in the presence and in the absence of serum. However, the addition of 5 per cent serum made the heart more resistant to the toxic actions of

all the inhibitors tested and therefore made it possible to test higher concentrations of the various agents.

Fluoroacetate appears to increase the sensitivity of all hearts to the blocking action of G-D 218 to a common level, irrespective of the season of the year or the temperature or hormone treatment of the animals (table 2).

Studies during March on hearts which could be blocked by relatively high doses of G-D 218 alone indicate a limited reciprocal relationship between this agent and fluoroacetate (table 5). Under these circumstances, a decrease in the concentration of G-D 218 could be compensated for by an increase in the concentration of fluoroacetate within limits. However, in the fully resistant summer-frog heart both G-D

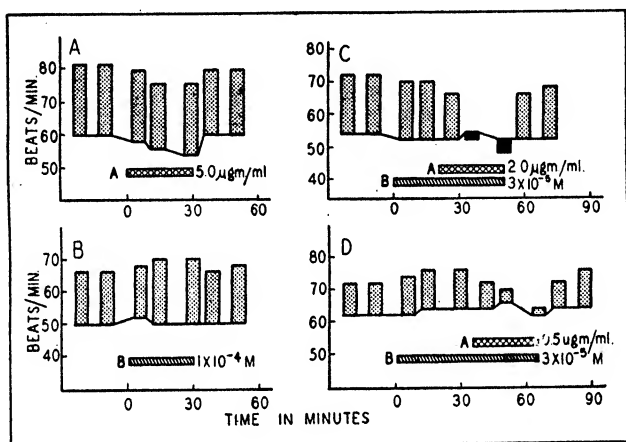


Fig. 3. BLOCKADE OF CHRONOTROPIC RESPONSE of the isolated summer-frog heart to epinephrine by G-D 218 plus fluoroacetate. Base line indicates control rate, and vertical columns indicate maximum change in rate in response to 1.0 µg. of epinephrine. A, G-D 218; B, fluoroacetate. Note that neither G-D 218 alone (A) nor fluoroacetate alone (B) is effective whereas combinations of the two, even in much lower concentrations, provide a complete blockade of the epinephrine-induced tachycardia (C and D).

218 and fluoroacetate were completely ineffective in all tolerated concentrations when tested alone (table 4).

In addition to the above experiments, a number of metabolic inhibitors were tested in combination with fluoroacetate in an effort to detect some other agent capable of blocking in essentially the same manner as the  $\beta$ -haloalkylamine and ergot adrenergic blocking agents. These attempts were completely unsuccessful, as illustrated by the results shown in the last column of table 4. Although many known metabolic inhibitors are not included in this series, the substances tested represent a reasonable cross-section of the various types of inhibitors and loci of action which have been studied. The fact that none of these agents was capable of combining its action with that of fluoroacetate to produce a blockade in the same manner as G-D 218 and dihydroergocornine indicates that these adrenergic blocking agents represent a distinct type of metabolic inhibitor. It is also of interest to note the very high dilu-

tion in which the active agents produce a blockade. Monofluoroacetate is effective in a concentration of  $3 \times 10^{-7}$  M and 0.2  $\mu\text{g/ml}$ . of G-D 218 represents a concentration of  $5.8 \times 10^{-7}$  M.

*Antagonism of the Blockade.* The activity of fluoroacetate, and particularly the fact that iodoacetate is active in producing a blockade whereas iodoacetamide is completely inactive, suggested that the metabolic pathways involved in supplying energy for the chronotropic response of the frog heart to epinephrine might involve acetate metabolism. Consequently glycerolmonoacetate (monoacetin) was employed

TABLE 4. BLOCKADE OF THE CHRONOTROPIC RESPONSE OF THE SUMMER-FROG HEART WITH VARIOUS INHIBITORS AND COMBINATIONS

AGENT	ALONE		WITH 5 $\mu\text{g/ml}$ . G-D 218		WITH $1 \times 10^{-4}$ M FLUOROACETATE	
	Conc. <sup>1</sup>	Result	Conc. <sup>1</sup>	Result	Conc. <sup>1</sup>	Result
G-D 218	10.0 $\mu\text{g/ml}$ .	o			0.2 $\mu\text{g/ml}$ .	Blockade
	2.0 $\mu\text{g/ml}$ .	o	2.0 $\mu\text{g/ml}$ .	o	0.2 $\mu\text{g/ml}$ .	o
Dihydroergocornine	3.0 $\mu\text{g/ml}$ .	Blockade	3.0 $\mu\text{g/ml}$ .	Blockade	0.5 $\mu\text{g/ml}$ .	Blockade
Sodium cyanide	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o
Sodium azide	$3 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o
Cadmium chloride	$1 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o
Sodium arsenite	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o
Sodium arsenate	$1 \times 10^{-3}$ M	o	$1 \times 10^{-3}$ M	o	$1 \times 10^{-3}$ M	o
Sodium fluoride	$3 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o
$\alpha, \alpha'$ -dipyridyl	$1 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o
2,4-dinitrophenol	$2 \times 10^{-4}$ M	o	$2 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o
Sodium monofluoroacetate	$1 \times 10^{-4}$ M	o	$3 \times 10^{-7}$ M	Blockade		
Potassium monoiodoacetate	$1 \times 10^{-4}$ M	o	$1 \times 10^{-5}$ M	Blockade	$5 \times 10^{-4}$ M	o
Iodoacetamide	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o
Sodium pyrophosphate	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o
Malonic acid	$1 \times 10^{-4}$ M	o	$1 \times 10^{-4}$ M	o	$5 \times 10^{-4}$ M	o

<sup>1</sup> Minimum effective concentrations of active blocking agents, or maximum concentrations in which inactive agents were tested.

in an effort to overcome the blockade after it had been established. Previous work by Chenoweth and co-workers (6) has indicated that this agent is effective in preventing the lethal action of fluoroacetate in mammals. This action of monoacetin is presumably due to the provision of 'utilizable acetate' at some point beyond the locus of blockade by fluoroacetate. Monoacetin was first tested on hearts blocked by a combination of fluoroacetate and G-D 218, and proved to be very effective in abolishing the blockade produced by these agents (fig. 4 and table 6). This antagonism is characterized by a rapid onset. Whereas the blockade produced by near threshold concentrations of G-D 218 or G-D 218 plus fluoroacetate usually requires 30 to 60 minutes to appear, the monoacetin antagonism usually appears within 5 minutes. Glycerol and sodium acetate were completely ineffective, even when tested in much

higher concentrations than those in which monoacetin rapidly eliminated the blockade.

In addition, the effect of added monoacetin on the blockade produced by G-D 218 or dihydroergocornine alone was tested. In these experiments also, monoacetin was very effective in eliminating the blockade (table 6). It thus appears probable that the  $\beta$ -haloalkylamines, the ergot alkaloids and fluoroacetate are all capable of blocking some phase of 'acetate' metabolism. Although the blockade produced may be in different metabolic pathways, it appears to affect a similar step in metabolism.

*Quantitative Aspects of the Blockade.* Quantitative data on the antagonism between epinephrine and dihydroergocornine or G-D 218 were obtained by testing the response of sensitive hearts to graded doses of epinephrine after perfusion for one hour with each of several increasing concentrations of the blocking agent. Although the exact maximum concentration of epinephrine to which the heart was exposed after each injection could not be determined, the small size of the tubing of the perfusion apparatus at the point of injection probably allowed little mixing. Any

TABLE 5. BLOCKADE OF CHRONOTROPIC RESPONSE OF THE MODERATELY RESISTANT FROG HEART BY FLUOROACETATE IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF G-D 218

TIME OF YEAR	CONC. G-D 218	MIN. EFFECTIVE CONC. FLUOROACETATE	TIME OF YEAR	CONC. G-D 218	MIN. EFFECTIVE CONC. FLUOROACETATE
	$\mu\text{g/ml.}$			$\mu\text{g/ml.}$	
March	5.0	0	March	1.	$3 \times 10^{-7} \text{ M}$
March	3.0	$2 \times 10^{-7} \text{ M}$	March	0.5	$5 \times 10^{-7} \text{ M}$
March	2.0	$3 \times 10^{-7} \text{ M}$	March	0.2	$5 \times 10^{-8} \text{ M}$

dilution which may have occurred in the tip of the cannula or in the ventricular chamber was probably essentially equal with the different doses of epinephrine, since they all involved the same volume of solution and rate of injection. In these experiments fluoroacetate in a concentration of  $1 \times 10^{-4}$  was added to all perfusion solutions in order to insure a uniform sensitivity of the hearts tested (see table 2). Averaged results of 6 experiments with G-D 218 and 4 experiments with dihydroergocornine are presented in figure 5.

Dihydroergocornine over a concentration range of 10-fold was found to block the response to epinephrine at a constant ratio of blocking to stimulating agent. These data indicate a competitive relationship between dihydroergocornine and epinephrine. The relationship between G-D 218 and epinephrine is clearly exponential. The difference between this relationship and that between dihydroergocornine and epinephrine is probably best explained on the basis of the stable reaction of the  $\beta$ -haloalkylamines with various tissue constituents during the production of the blockade (3) rather than on the assumption of a completely different locus of action.

Quantitative data regarding the antagonism between fluoroacetate and epinephrine were obtained in experiments carried out in the presence of 5  $\mu\text{g/ml.}$  of G-D 218 on hearts made completely resistant to the latter agent by injecting the frogs with anterior pituitary extract 24 hours prior to use. The results are presented in

figure 5 and indicate that blockade of the chronotropic response to epinephrine by fluoroacetate is competitive.

The concentration of monoacetin required to antagonize the blockade produced by varying doses of G-D 218 and fluoroacetate was also determined. Studies with G-D 218 alone were carried out on hearts which were highly sensitive to blockade by this agent, and those with fluoroacetate were performed in the presence of  $5 \mu\text{g}/\text{ml}$  of G-D 218 on highly resistant hearts whose chronotropic response to epinephrine was not blocked by any tolerated concentration of G-D 218 alone. The concentration of glycerolmonoacetate required to abolish the blockade was surprisingly constant. These experiments clearly demonstrate that antagonism of the blockade by this agent is non-competitive (table 7). In all experiments monoacetin behaved as a 'product' of the reactions blocked. It appears to supply a utilizable 'metabolite' at some point beyond the blockade; this situation is comparable to that postulated

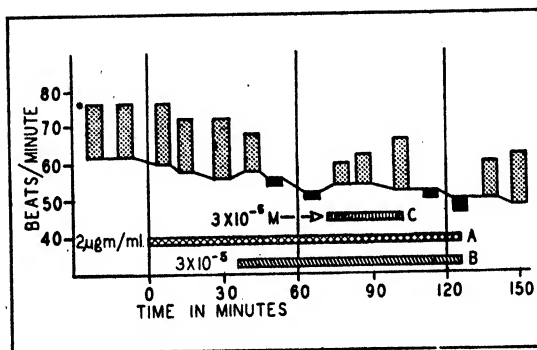


Fig. 4. BLOCKADE OF CHRONOTROPIC RESPONSE of the isolated summer-frog heart to epinephrine by G-D 218 and fluoroacetate and antagonism of the blockade by glycerolmonoacetate. Base line indicates control rate and vertical columns indicate maximum change in rate in response to  $1.0 \mu\text{g}$  of epinephrine. Note that the addition of glycerolmonoacetate completely abolishes the blockade produced by these agents. A, G-D 218; B, fluoroacetate; C, glycerolmonoacetate.

to explain its antagonism of fluoroacetate toxicity in mammals (6). However, this postulate has not previously received quantitative confirmation.

Monoacetin does not alter the inotropic response to epinephrine, nor does it improve the rate or amplitude of contraction of hearts which have become hypodynamic either because of prolonged perfusion with frog-Ringer solution without serum or because of perfusion with high concentrations of various inhibitors.

Although the characteristics of their antagonism by monoacetin are the same, the blockade produced by the typical adrenergic blocking agents G-D 218 and dihydroergocornine and by fluoroacetate must occur in different metabolic systems, because neither the adrenergic blocking agents alone nor fluoroacetate alone is capable of altering the chronotropic response of the summer-frog heart. Both of these systems appear to supply 'utilizable acetate' or some similar source of energy to a common pool from which the metabolic energy for the chronotropic response to epinephrine is derived.

#### DISCUSSION

On the basis of the data presented above, it can be stated unequivocally that certain adrenergic blocking agents are capable of inhibiting the chronotropic response

of the frog heart to epinephrine, norepinephrine and isopropyl-norepinephrine. This action appears to be an expression of specific adrenergic blocking activity similar to the inhibition of excitatory responses of smooth muscle. The necessary concentrations of various blocking agents are comparable to those required to inhibit smooth muscle responses, and the blocking agents tested exhibit essentially the same relative potencies by the two types of tests. In addition, ergonovine and several congeners of Dibenamine, which are ineffective against smooth muscle responses to epinephrine, also fail to block this chronotropic response. However, the production of this blockade is highly dependent upon the condition of the experimental animal. It

TABLE 6. ANTAGONISM OF THE BLOCKADE PRODUCED BY G-D 218, DIHYDROERGOCORNINE OR G-D 218 PLUS FLUOROACETATE

TIME OF YEAR	BLOCKING AGENT	CONC.	'METABOLITE'	CONC. <sup>1</sup>	RESULT
		$\mu\text{g/ml.}$			
Nov.	G-D 218	1.0	Glucose	0.1-1.0%	o
Dec.	G-D 218	1.0	Glycerolmonoacetate	$4 \times 10^{-6}$ M	Blockade abolished
Jan.	G-D 218	1.0	Sodium acetate	$5 \times 10^{-4}$ M	o
Jan.	G-D 218	1.0	Glycerol	$5 \times 10^{-4}$ M	o
Oct.	G-D 218	0.5	Glycerol monoacetate	$4 \times 10^{-6}$ M	Blockade abolished
	Fluoroacetate	$1 \times 10^{-6}$ M			
Oct.	G-D 218	0.5	Sodium acetate	$5 \times 10^{-4}$ M	o
	Fluoroacetate	$1 \times 10^{-6}$ M			
Oct.	G-D 218	0.5	Glycerol	$5 \times 10^{-4}$ M	o
	Fluoroacetate	$1 \times 10^{-6}$ M			
Jan.	Dihydroergocornine	1.0	Glycerol monoacetate	$4 \times 10^{-6}$ M	Blockade abolished
Jan.	Dihydroergocornine	1.0	Sodium acetate	$1 \times 10^{-4}$ M	o
Jan.	Dihydroergocornine	1.0	Glycerol	$1 \times 10^{-4}$ M	o

<sup>1</sup> Minimum effective concentrations of active agents, or maximum concentrations in which inactive agents were tested.

appears probable that many of the failures to produce blockade which have been reported in the past (1) may be attributed to the season of the year in which the experiments were performed.

The difference between the seasonal changes in sensitivity to the blocking action of the  $\beta$ -haloalkylamines (G-D 218) and the ergot alkaloids (dihydroergocornine) can probably be best explained on the basis of the direct cardiac depressant actions of the latter (1). Failure of the summer-frog heart to become completely resistant to dihydroergocornine (fig. 2) may be explained on the assumption that G-D 218 and dihydroergocornine have a primary action or site of action in common, but that the ergot alkaloid is producing some additional depression of the heart action. The essentially simultaneous seasonal changes in the response to the two agents, their common synergism with fluoroacetate and antagonism by monoacetin, and the



comparable changes in sensitivity induced by cold and pituitary extracts strongly suggest that the primary blocking action of the ergot alkaloids occurs at a site very similar to that affected by members of the Dibenamine series of blocking agents.

Since the observation of Reid in 1890 that the skin of frogs after the spring breeding season has less ability to effect the active transport of water than the skin of winter frogs (7), a large number of scattered reports have indicated differences between summer and winter frogs in several physiological properties as well as in their

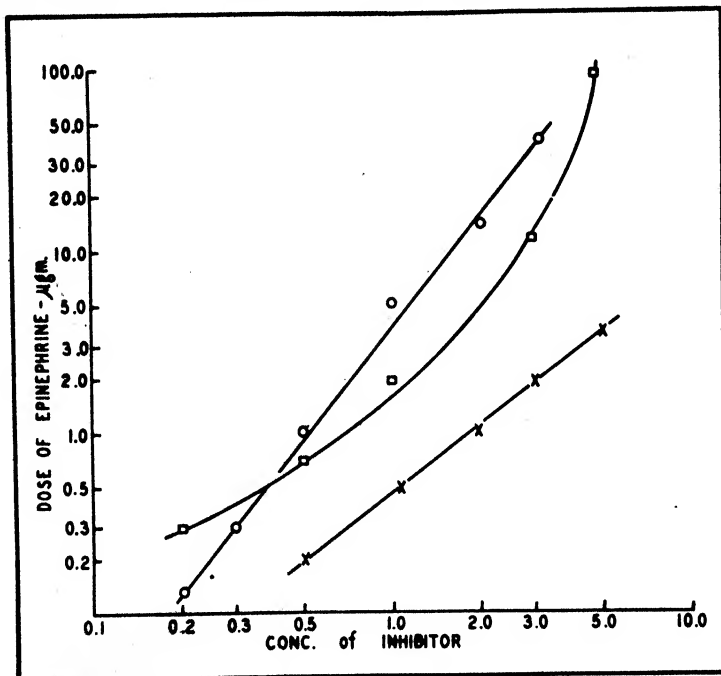


Fig. 5. QUANTITATIVE RELATIONSHIPS of epinephrine and various inhibitors in blockade of the chronotropic response of the isolated frog heart. Circles represent fluoroacetate, concentration  $\times 10^{-6}$  M; squares represent G-D 218, concentration in  $\mu\text{g./ml.}$ ; X marks represent dihydroergocornine, concentration in  $\mu\text{g./ml.}$

responses to a variety of drugs. The limited data provided in most of these reports do not allow any evaluation of their relationship to the phenomena under consideration here and consequently only a few of them are mentioned. It has been noted that the extracellular calcium level is higher (8), that the myocardial carbohydrate stores are lower (9, 10) and that the carbohydrate utilization of the beating heart is higher (10) in summer than in winter frogs. Barcroft and Izquierdo (11) observed that the rate of the summer-frog heart varies in a linear fashion with the reciprocal of the absolute temperature, whereas the logarithm of the rate of the winter-frog heart is a linear function of the reciprocal of the absolute temperature. Their data on the winter-frog heart are in general agreement with earlier observations of Barlow and

Sollmann (12). The present results offer a possible explanation of this difference in response which is discussed in more detail below.

Hofmann (13) has recently demonstrated, on the basis of impedance measurements, that the permeability of the skin, cardiac muscle, stomach mucosa and cloacal mucosa in *R. esculenta*, is much higher in the summer than in the winter. In the case of skin, which was most carefully studied, the increase in permeability, beginning in February, closely parallels the increased resistance to adrenergic blockade demonstrated in the present experiments. This difference is related to the environmental temperature in that it can be prevented by keeping the animals in the cold. However, the development of increased permeability is independent of temperature once it is initiated, to the extent that it progresses at a reduced rate despite cooling.

TABLE 7. GLYCEROLMONOACETATE ANTAGONISM OF BLOCKADE PRODUCED BY VARIOUS CONCENTRATIONS OF G-D 218 AND FLUOROACETATE

TIME OF YEAR	TREATMENT OF PROGS	CONC. OF BLOCKING AGENT	CONC. GLYCEROLMONOACETATE	
			Effective	Ineffective
<i>G-D 218 alone</i>				
Dec.	25 days at 8°C.	0.2 µg/ml. (5.8 × 10 <sup>-7</sup> M)	4 × 10 <sup>-6</sup> M	3 × 10 <sup>-6</sup> M
Dec.	25 days at 8°C.	1.0 µg/ml. (2.9 × 10 <sup>-6</sup> M)	4 × 10 <sup>-6</sup> M	3 × 10 <sup>-6</sup> M
Dec.	25 days at 8°C.	5.0 µg/ml. (1.5 × 10 <sup>-5</sup> M)	4 × 10 <sup>-6</sup> M	3 × 10 <sup>-6</sup> M
<i>Fluoroacetate plus 5 µg/ml. G-D 218</i>				
Feb.	Ant.-pit. extr.	1 × 10 <sup>-6</sup> M	4 × 10 <sup>-6</sup> M	3 × 10 <sup>-6</sup> M
Feb.	Ant.-pit. extr.	1 × 10 <sup>-6</sup> M	4 × 10 <sup>-6</sup> M	3 × 10 <sup>-6</sup> M
Feb.	Ant.-pit. extr.	1 × 10 <sup>-4</sup> M	4 × 10 <sup>-6</sup> M	3 × 10 <sup>-6</sup> M

Maximum permeability was noted in August and the seasonal decrease in permeability began about one month later than the return of sensitivity to blockade by G-D 218 noted above.

Although these two processes are essentially parallel, caution must be observed in attributing a causal relationship to this coincidence. Data from our laboratory, which will be reported elsewhere, indicate that increased cellular permeability, induced by reducing the calcium content of the perfusate or by adding certain surface active agents, is associated with an increased rather than a decreased sensitivity to blockade. It appears most probable that both phenomena are expressions of the same underlying factor which, on the basis of our experiments, appears to be a seasonal variation in anterior pituitary activity.

The response of the heart to vagus and accelerator nerve stimulation varies widely at different seasons of the year, but various species appear to have different cycles of sensitivity and resistance (14). In addition, several observations have indicated seasonal variations in responses to exogenous epinephrine. Peripheral

vasoconstriction has been reported to be increased in the winter in intact animals (15) and to be decreased in the winter in perfused preparations (16). The basis for this difference is not apparent; *R. temporaria* was employed in parts of both studies. It is possible that the differences are related to variations in the composition of the circulating media. Epinephrine is known to lose much of its vasoconstricting activity on the perfused vessels of the frog leg when calcium is reduced or absent (17). The frog heart has also been reported to be more sensitive to epinephrine in the summer than in the winter (18), although other workers have failed to find a significant seasonal variation in the response of the hypodynamic heart to this agent (19). Even the mammalian (guinea pig) heart has been reported to be more sensitive to epinephrine in the summer (20). These and many other scattered observations indicate that summer frogs are indeed quite different from winter frogs in their physiological and pharmacological responses. However, the available data are not adequate to

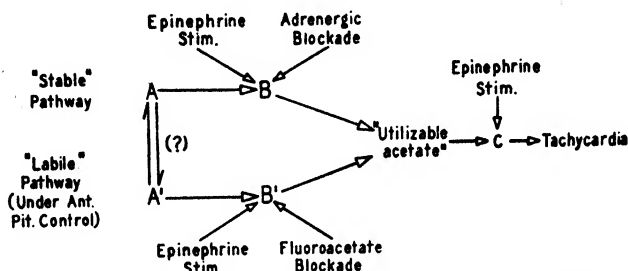


Fig. 6. POSSIBLE POINTS OF ACTION of epinephrine and certain blocking agents on the processes leading to the production of an epinephrine-induced tachycardia. *A* and *A'* represent metabolic substrates in the myocardium which may or may not be independent of each other. *B* and *B'* are parallel reactions affected by epinephrine and blocking agents. *C* depicts the final point of action of epinephrine in initiating the tachycardia.

allow any clear evaluation of the basis for these differences or of their relationship to the present observations.

In our experiments the differences between the responses of the heart of summer and winter frogs appear to be under the control of the anterior pituitary gland. Earlier workers such as Reid (7) and Karasek and Poupa (15) correlated changes in water transport through the skin and changes in the peripheral vascular response to epinephrine with the breeding season. The very rapid increase in resistance to blockade shown in figure 2 occurs shortly before the beginning of the breeding season of *R. pipiens* in the area from which our animals were obtained. In addition, the marked increase in resistance induced by the injection of whole- or anterior-pituitary extracts provides direct evidence that the anterior pituitary gland is responsible for the changes in sensitivity to adrenergic blockade. However, at the present time it is not clear what fraction of the anterior-pituitary extract is involved or whether certain target glands must act as intermediaries. Failure to obtain an effect from pituitary extract added to the perfusate suggests that the pituitary effect is mediated through other glands, but the duration of such experiments is limited and other interpretations are possible. This problem is now being investigated further.

The positive chronotropic response of the heart to epinephrine is an expression of an increased production and/or utilization of energy. Failure of Dibenamine and its congeners to block this release of energy in the summer-frog heart suggested that some pathway in addition to the one responsible for the release of energy in the winter-frog heart might be involved. This interpretation has proved to be correct. Although the  $\beta$ -haloalkylamines are completely ineffective in blocking the chronotropic response of the summer-frog heart, these agents combine with low concentrations of fluoroacetate or iodoacetate to inhibit the response of the summer-frog heart in almost exactly the same way, and in the same concentration, that the blocking agent alone inhibits the response of the winter-frog heart. This indicates that two quite distinct mechanisms are involved in the production of the tachycardia in response to epinephrine. Either of these mechanisms appears to be adequate to allow a normal response to epinephrine in the summer-frog heart. One of these processes, that blocked by the  $\beta$ -haloalkylamines and ergot alkaloids, appears to be quite constant throughout the year and under various conditions of temperature and anterior-pituitary activity, i.e. the concentration of G-D 218 necessary to produce a block in the presence of fluoroacetate is quite constant (table 2). This may be referred to as the 'stable' mechanism or pathway. The second pathway is blocked by fluoroacetate and iodoacetate and varies from almost complete inactivity during the winter months, to activity capable of sustaining the entire response during the late spring and summer. As noted above this 'labile' mechanism appears to be under the control of the anterior pituitary gland.

The experiments presented above allow certain conclusions regarding the site and mode of action of the  $\beta$ -haloalkylamines and the ergot alkaloids. In addition, they throw considerable light on the mechanism by which epinephrine induces cardioacceleration. Although epinephrine was the first hormone to be isolated, almost nothing has been known regarding the mechanism by which it stimulates smooth or cardiac muscle.

The process inhibited by the adrenergic blocking agents appears to be some step in the production of 'utilizable acetate' or other 2-carbon fragment. This is clearly indicated by the non-competitive manner in which the blockade is antagonized by monoacetin. A constant amount of the 'product' of the reactions blocked is necessary to allow stimulation of the heart by epinephrine, irrespective of the amount of blocking agent present above the minimum required for blockade.

The clear-cut non-competitive antagonism of the blockade produced in sensitive hearts by the adrenergic blocking agents alone by an 'acetate donor,' monoacetin, provides convincing evidence that these agents are inhibiting the production of some metabolite which subserves the same functions as 'utilizable acetate.' Unfortunately, the blocking action of fluoroacetate or iodoacetate alone cannot be studied on the frog heart. However, several points of evidence strongly suggest that fluoroacetate and iodoacetate are acting in the 'labile' pathway on a metabolic step similar to that inhibited by the adrenergic blocking agents in the 'stable' process. These are as follows:

- 1) Previously reported studies on the pharmacology of fluoroacetate indicate that it inhibits some phase of acetate metabolism. The exact locus of this blockade

is still open to question, but the data obtained in our experiments are compatible with the conclusion of Bartlett and Barron (21) that "Fluoroacetate probably acts by inhibiting the formation of 'active' acetate (the so-called  $C_2$  compound, which may be an acetyl derivative or an acetate radical)."

2) Iodoacetate, but not iodoacetamide, is effective in producing the blockade. This suggests that the structural similarity to acetate rather than the sulfhydryl-alkylating property of the iodoacetate is important. On the basis of convincing evidence it has been stated (22) that "There is no longer any reason to believe that fluoroacetate is in any way pharmacologically analogous to iodoacetate." Our experiments do not suggest that fluoroacetate is acting in a manner similar to the classical action of iodoacetate, i.e. by the alkylation of sulfhydryl groups. However, they do indicate that iodoacetate, presumably before release of the iodine, may have an action similar to that of fluoroacetate. The relatively rapid loss of iodine from the iodoacetate may be a factor in its definitely lower activity when compared to fluoroacetate.

3) During periods of intermediate sensitivity to G-D 218, there is a reciprocal relationship between the concentration of G-D 218 and the simultaneous concentration of fluoroacetate required to produce a blockade (table 5). This observation indicates that the products of the two reactions blocked are the same, or at least serve the same metabolic function.

4) The blockade produced by fluoroacetate in the presence of G-D 218 is antagonized by monoacetin in a non-competitive manner. This is not conclusive evidence as in the case of G-D 218, because of the simultaneous involvement of the 'stable' system, but if the fluoroacetate were interfering with the utilization of 'acetyl' or acting on some system unrelated to the production of 'utilizable acetate,' one might expect this clear relationship to be distorted.

5) Various doses of epinephrine are antagonized by fluoroacetate, in the presence of a complete blockade of the 'stable' system, in a competitive manner comparable to the antagonism of epinephrine by dihydroergocornine (fig. 5).

The competitive nature of the inhibition of the epinephrine response by dihydroergocornine, G-D 218 and fluoroacetate has been illustrated in figure 5. The  $\beta$ -haloalkylamines appear to have the same locus of blockade as other adrenergic blocking agents in the cases which have been studied (23), and the exponential rather than linear relation observed with G-D 218 appears to be best explained on the basis of the stable reactions between the  $\beta$ -haloalkylamines and tissue constituents during the production of the blockade (3) rather than by assuming different loci of action for the  $\beta$ -haloalkylamines and ergot alkaloids.

On the basis of the aforementioned observations, it appears that epinephrine, must be acting at two distinct points in the energy metabolism of the frog myocardium to promote the production of utilizable acetate or some suitable substitute. The competitive nature of the antagonism of the epinephrine response by both adrenergic blocking agents and fluoroacetate indicates that these agents are indeed blocking a specific action of the epinephrine rather than merely inhibiting some related process necessary for subsequent stimulation by epinephrine.

In addition to its effect in producing utilizable acetate, epinephrine exerts what

may be referred to as a 'trigger action.' The administration of monoacetin does not in itself produce an increase in the heart rate. It merely provides a necessary substrate for the release of energy for the chronotropic response. The final effect is dependent on the simultaneous presence of this substrate and epinephrine.

On the basis of the above data and discussion, the sites of action of epinephrine, the  $\beta$ -haloalkylamine or ergot alkaloid blocking agents, and fluoroacetate or iodoacetate in producing or inhibiting the chronotropic response of the frog heart may be tentatively diagrammed as in figure 6. No evidence is available at the present time to indicate whether the two substrates *A* and *A'* are identical or metabolically related. The adrenergic blocking agents of the  $\beta$ -haloalkylamine and ergot alkaloid series act to block the stable pathway. Fluoroacetate and iodoacetate block in essentially the same manner the labile pathway. In addition to promoting the production of utilizable acetate through these two pathways, epinephrine stimulates some final trigger mechanism which causes the release of energy in the form of a tachycardia if a suitable substrate is present.

Evidence for the presence of at least two steps in the process by which epinephrine causes an increase in cardiac rate offers an intriguing explanation for the observations of Barcroft and Izquierdo (11) on the effect of temperature on heart rate in winter and summer frogs. They noted that the rate of the summer-frog heart varies in a linear fashion with the reciprocal of the absolute temperature, whereas the logarithm of the rate is a linear function of the reciprocal of the absolute temperature in the winter-frog heart. Epinephrine release is not directly involved in the heat-induced cardioacceleration because this response is not altered by high concentrations of ergotamine (24) even during October and November, a period of relative sensitivity to blockade. However, if mechanisms similar to those demonstrated for the epinephrine stimulation are involved in the thermal stimulation, it may be postulated that in the summer-frog heart, which has two mechanisms for the production of the necessary substrate, the final 'triggering' step is the limiting reaction. The relationship to temperature would thus be a direct one. However, in the winter-frog heart, with a lower ability to produce substrate, the production of utilizable acetate may be the limiting step. Thus the relationship between rate and temperature would be expected to be logarithmic.

The process of blockade by the adrenergic blocking agents studied appears to be quite different from the inhibition and reversal of the epinephrine response by phenolases reported by Heirman (25) and Bacq (26, 27). These workers observed that inhibition of the inotropic response was equal to or greater than inhibition of the chronotropic response, whereas we observed the inotropic response to epinephrine to be unaffected by any of the blocking agents or combinations of agents under conditions which completely eliminated the chronotropic response. These workers also found that prior treatment of the epinephrine solution with phenolases produced the same effect as exposing the heart to the enzymes; and interpreted their data as indicating that a chemical change in the mediator was responsible for the altered response. Tiffeneau and Beauvallet (28) noted a similar change in the epinephrine response after prolonged exposure of the heart to a small volume of epinephrine solution. However, chemical alteration of the mediator as an explanation of the action of

Dibenamine and its congeners is excluded by the slow onset of blockade during continuous infusion of the blocking agent in the absence of epinephrine, and by previously reported experiments demonstrating the failure of Dibenamine to alter the activity of epinephrine after prolonged *in vitro* incubation (5).

Krayer (29, 30) and Krayer and van Maanen (31) have recently reported blockade by veratrum alkaloids of the chronotropic but not of the inotropic effects of epinephrine in heart-lung preparations and in intact dogs. However, no further analysis of the mechanism of action of these agents has been reported, and it is impossible at the present time to evaluate the relationship of their observations to the blockade produced by adrenergic blocking agents, with or without fluoroacetate, on the frog heart. The relationship between these two phenomena is currently being investigated.

An additional point of interest in the present observations is the apparent specificity of the energy sources necessary to support the chronotropic and inotropic responses to epinephrine. The two pathways for the production of utilizable acetate which have been demonstrated appear to be involved in supplying energy for the chronotropic response only. Lichneckert and Straub (19) have recently observed that adenosinetriphosphate in very high dilution will revive the hypodynamic summer-frog heart, but is completely ineffective in the winter-frog heart. On the basis of a similarity between the action of ATP and epinephrine, they have suggested that the increase in the strength of contraction of the hypodynamic heart induced by epinephrine may be due to catalysis of some process involving adenosinetriphosphate. Up to the present time, we have been unable to demonstrate a relationship of ATP to the chronotropic response of the heart to epinephrine, and the records pictured by the above authors indicate that recovery of the strength of contraction in response to ATP is not accompanied by an increase in rate.

Although the presence of specific energy sources for specific physiological functions has frequently been postulated, convincing evidence for such a dissociation in vertebrate tissues has been presented in few cases, and in no instance have the exact metabolic pathways involved been elucidated. Spratt demonstrated that the early chick blastoderm may continue to differentiate in the absence of amino acids, but that growth is inhibited (32). He also observed that most metabolic inhibitors prevent development of the central nervous system without preventing development of the heart, but that fluoride has just the opposite specificity (33). Gerard and Doty (34) demonstrated a dissociation between the oxygen consumption associated with nerve activity (blocked by azide) and that associated with resting metabolism (blocked by methylfluoroacetate), but these workers have not yet analyzed the metabolic processes involved. Unfortunately, in these and most other studies demonstrating a dissociation of metabolic activities subserving different functions, growth and/or total oxygen consumption have been employed as criteria. These complex phenomena have not yet lent themselves to a precise delineation of the basic metabolic factors responsible for the over-all differences observed.

#### SUMMARY

A study of the responses of the isolated, perfused frog heart to epinephrine (and to norepinephrine and isopropylnorepinephrine) has revealed the following: 1) The

chronotropic response of the winter-frog heart is effectively blocked by low concentrations of several  $\beta$ -haloalkylamines and ergot alkaloids. The blockade produced by the  $\beta$ -haloalkylamines in this preparation is not persistent as in the case of the blockade of mammalian smooth muscle responses to adrenergic stimuli. 2) The chronotropic response of the summer-frog heart is unaltered by the  $\beta$ -haloalkylamines and the ergot alkaloids have much less effect than on the winter-frog heart. Resistance to blockade develops rapidly in early March and sensitivity returns late in August. This seasonal difference is not closely correlated with either the environmental temperature or the temperature at which the experiments are conducted. 3) Sensitivity can be induced in resistant animals, at least during the last part of the resistant period, by exposing the animals to low temperatures for several days. Exposing the animals to low temperatures for only a few days or cooling the perfusate does not produce a similar effect. 4) Anterior-pituitary extract injected into the intact animal decreases the sensitivity of the winter-frog heart to blockade so that it is comparable to the summer-frog heart. Anterior-pituitary extract is ineffective when added to the perfusion solution. 5) Although completely ineffective alone, fluoroacetate or iodoacetate, combined with a  $\beta$ -haloalkylamine or ergot alkaloid, produces a blockade of the resistant, summer-frog heart comparable to that produced by the adrenergic blocking agents alone in the winter-frog heart. Many other metabolic inhibitors were tested and all were ineffective alone or combined with  $\beta$ -haloalkylamines or fluoroacetate. 6) Blockade of the chronotropic response to epinephrine by the adrenergic blocking agents, or by fluoroacetate in the presence of an ineffective concentration of a  $\beta$ -haloalkylamine, has the characteristics of a competitive reaction. 7) Glycerolmonoacetate antagonizes the blockade produced by the adrenergic blocking agents alone, or combined with fluoroacetate, in a non-competitive manner. It behaves strictly as a 'product' of the blocked reaction. Glycerol and sodium acetate are ineffective. The blocked heart is not stimulated to an increased rate of contraction by the presence of glycerolmonoacetate alone, but the presence of this substance in low concentrations does allow a normal response to epinephrine.

It is concluded that the chronotropic response of the frog heart to epinephrine is dependent upon two effects of the activating agent. 1) A stimulation of the production of utilizable acetate, and 2) a trigger action which is effective only in the presence of a suitable acetyl substrate. The epinephrine-stimulated production of utilizable acetate may proceed by two distinct pathways—a stable pathway which appears to be qualitatively and quantitatively constant throughout the year and which is blocked by  $\beta$ -haloalkylamine and ergot alkaloid adrenergic blocking agents, and a labile pathway which normally functions only in the summer-frog heart, which is activated by the anterior pituitary gland and which is blocked by fluoroacetate or iodoacetate. Both of these pathways appear to be independent of the energy sources utilized in the normal contraction of the heart and in its positive inotropic response to epinephrine. Epinephrine thus appears to promote the production of the metabolic substrate required for the expression of its own chronotropic action.

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# EFFECTS OF DIPHENYLHYDANTOIN SODIUM (DILANTIN SODIUM) AND PHENOBARBITAL SODIUM UPON ECTOPIC VENTRICULAR TACHYCARDIA IN ACUTE MYOCARDIAL INFARCTION<sup>1</sup>

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THE occlusion of the anterior descending ramus of the left coronary artery of the dog's heart by a two-stage technique (1) results in the survival of all animals through the immediate post-occlusion period, and in the development of an ectopic ventricular tachycardia which begins after a delay of  $4\frac{1}{2}$  to 8 hours. During this latent period low frequency (1 to 5/min.) ectopic ventricular beats may occur. Sporadic higher frequencies have been observed in a few experiments prior to the main rise. The onset of the main rise is rather abrupt, and the rate increases rapidly during the next 3 or 4 hours and then more slowly, reaching a maximum of 160 to 260 per minute after 8 to 20 hours following occlusion. Ectopic activity ceases in untreated animals within 2 to 5 days. Usually there are 1 to 3 days during which tests of the effects of drugs upon ectopic rhythms can be performed. Rarely, an animal fails to develop sufficiently rapid or sufficiently continuous ectopic discharges to be considered acceptable for use in drug testing.

The latency of  $4\frac{1}{2}$  to 8 hours before the rapid development of ectopic activity approximates the minimal duration of ischemia that is required to produce histologic signs of necrosis (2,3). Upper thoracic sympathectomy has been found to have little effect upon this latency. This fact together with the correspondence in timing suggests the principal ectopic excitation may be associated with necrosis.

Evidence that the *early* ectopic ventricular impulses that occur within a few minutes after abrupt occlusion of a coronary artery arise in the partially ischemic tissues of a narrow boundary surrounding the potential infarct has been presented previously (4,5). The central portion of the ischemic area quickly becomes inactive electrically when local bipolar leads (6) are used. Such electrodes favorably placed in the boundary record distorted complexes with elevated S-T tracings and exceptionally tall spikes. Within this thin boundary also must exist all degrees of ischemia including the moderate intensities that produce hyperexcitability (7). In chronic experiments the severe necrosis found in the zone of infarction and the entirely normal appearance

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<sup>2</sup> Jesse H. and Mary Gibbs Jones Scholar.

of the muscle found elsewhere upon sacrifice of animals indicate that the boundary is the most probable source of delayed ectopic impulses also.

In focal epilepsy associated the post-traumatic scars and expanding lesions the epileptogenic region of tissue is found in the boundary between the lesion and the histologically normal cortex. The epileptogenic region characteristically gives rise to spontaneous spike discharges, corresponds in localization to that from which the clinical seizures seem to arise as judged by pattern of onset, and is the only region at which the application of electric stimulation evokes seizures that are typical of the spontaneous ones or evokes reactions that are similar to the first elements of a spontaneous seizure (8,9). Surgical excision of such regions results in cessation of seizures in a significant percentage of cases. Histological examination of excised epileptogenic tissues reveals a marked diminution in a number of small blood vessels, and evidence of *progressive acute necrosis* in each of them (8,10).

The apparent similarities between the origins of delayed cardiac ectopic discharges following coronary occlusion and focal epileptogenic discharges suggested that drugs that have proved effective in preventing focal seizures might suppress ectopic ventricular discharges which accompany acute myocardial infarction. To test this possibility diphenylhydantoin sodium and phenobarbital sodium have been administered singly and in combination to dogs during delayed ectopic ventricular tachycardia following coronary occlusion.

#### METHODS

Under pentobarbital sodium or morphine-barbital sodium anesthesia and with artificial respiration, the anterior descending arteries of dogs' hearts were ligated aseptically via a small incision in the fourth intercostal space on the left side. The artery was freed from connective tissue and accompanying veins for a few mm. at a level 2 to 5 mm. distal to the margin of the left auricular appendage. A doubled ligature was passed under the artery and cut, thereby becoming two ligatures. One of the ligatures then was tied snugly but not tightly around the artery together with a no. 20 hypodermic needle. The needle was withdrawn immediately leaving the artery constricted but not closed. The second ligature was tightened 30 minutes later totally occluding the artery. By such occlusion in two stages loss of animals by early ventricular fibrillation has been prevented. The chest wound was closed in layers and natural respiration restored.

Electrocardiographic observations and records were made at frequent intervals. Ventricular ectopic rates and total heart rates were measured from electrocardiograms and plotted on charts. When ectopic discharges were few and intermittent, especially during the development of ectopic rhythms, and during drug tests when the ectopic frequency had been reduced greatly, the moving element of the electrocardiograph was observed for long periods and counts of ectopic frequencies were made.

Drug tests were begun in almost all animals after the passage of sufficient time to allow full recovery from the anesthetic used for surgery. Many of the animals were able to walk about the room and had an appetite for milk.

The administration of drugs for the determination of their effectiveness in the

suppression of ectopic discharges was by intravenous injection in a majority of trials. Injections were made only after continuous ectopic activity of considerable frequency had developed. Confidence in the interpretation of records was gained by the observation of quick diminution of frequency of ectopic complexes during and immediately after injection (dilantin) and by their return later. In the usual case, a single dose of the drug under test was insufficient to produce a significant reduction of ectopic frequency lasting longer than 10 to 30 minutes. Because of this a test usually consisted of multiple doses.

#### RESULTS

*Diphenylhydantoin Sodium (Dilantin Sodium)*.<sup>3, 4</sup> The results of 14 tests of the effects of dilantin alone in 10 dogs with active ventricular ectopic rhythms after all effects of the anesthetic used for surgery had disappeared are shown in condensed form in table 1. It was possible to control all ventricular tachycardias by adequate amounts of dilantin. The highest ectopic frequencies, 180 per minute and above, were found to require repeated doses until the amounts given totalled from 125 to 200 mg. per kg. to reduce the frequency of ectopic beats to zero and to keep it at a level less than one-half that which existed before the first dose for a period of 5 hours or longer. Smaller amounts, 75 and 100 mg. per kg. administered to two dogs with ectopic frequencies of 190 and 250, respectively, reduced the ectopic rates to a low level for 1 hr. 10 min. and 1 hr. 30 min., but the ectopic rates rose rapidly after these periods. In the animals with ectopic rates of 170 and below, dilantin in total amounts of 100 mg. per kg. and less has proved sufficient to keep ectopic activity at a low level for 5 hours or longer. There appears to be a rough proportionality between the frequency of ectopic impulses before treatment and the amount of dilantin that is required to suppress them for a given period of time.

Certain significant observations such as the temporary suppression of ectopic discharges by single doses and the more lasting effects of accumulated doses cannot be shown in a simple table. Figure 1 shows a dramatic conversion of a rapid ectopic rhythm to a sinus rhythm during an injection of dilantin. Such quick but often temporary conversions during injection were not uncommon. The charts which follow portray the relation that exists between amount of dilantin administered over a period of time and its duration of suppressor effect upon an intense ectopic excitatory state.

Figure 2 shows the effects of individual and cumulative doses of intravenous dilantin in *experiment C49* (table 1). When the first dose was injected, the dog was wide awake, friendly and willing to eat and drink. Twenty hours had elapsed since the occlusion. It can be seen that on the first post-occlusion day (17-30 hours) when the pre-treatment ectopic rate was 200 to 220 as much dilantin as 200 mg. per kg. was required to produce suppression with a duration lasting into hours, but that on the fourth post-occlusion day (66-74 hours) when the ectopic rate was 130 to 140 prior to treatment only one-half as much dilantin (100 mg/kg.) was required. No testing was done on the second post-occlusion day (records at 41 and 53 hours).

<sup>3</sup> For brevity the terms 'dilantin' and 'phenobarbital' are used instead of diphenylhydantoin sodium and phenobarbital sodium except in the title, subtitles and summary.

<sup>4</sup> The dilantin used in this study was generously supplied by Parke, Davis & Co.

An experiment which illustrates other features of the effects of dilantin is charted in figure 3. Smaller doses were used (25 mg/kg.). When the first dose was given 10½ hours after occlusion the ectopic rate was 190 to 200. Each injection produced temporary suppression of ectopic beats, but the total of the three doses given that night, 75 mg. per kg., was insufficient to produce prolonged suppression. On the following

TABLE I. EFFECTS OF DILANTIN SODIUM UPON VENTRICULAR ECTOPIC FREQUENCY

EXPER. NO.	AMT., MG./KG.	ECTOPIC FREQUENCY		DURATION REDUCTION TO ½	REMARKS
		BEGINNING	MIN.		
C-48	100 i.p.	110	0	7 hr.	1½ hr. after last (3rd) injection to reach 0. Maximal return ectopic rate 55.
C-49	200 i.v.	200	0	5 hr.	After 5 hr. another dose, 50 mg/kg. returned ectopic freq. to 0
C-49	100 i.v.	140	0	6 hr. 15 min.	1st 50 mg/kg. dose reduced ectopic freq. to 0 for 30 min.
C-53	100 i.v.	250	10	1 hr. 10 min.	During period when ectopic freq. usually is increasing.
C-56	75 i.v.	190	0	1 hr. 30 min.	During period when ectopic freq. usually is increasing.
C-56	100 i.v.	170	0	7 hr. +	7 hr. to 1st return of ectopic beats. Maximal return ectopic rate 30.
C-60	25 i.v.	140	0	2 hr. 20 min.	Nembutal 10 mg/kg. and morphine 10 mg. 4 hr. before and morphine 8 mg. 1 hr. before.
C-60	10 i.v.	30-210	0	Indef. +	During last 1½ hr. before this injection ectopic rate varied between 30 and 210 (brief paroxysm 210)
C-61	195 i.v.	212	0	15 hr. +	Maximal return ectopic rate 70.
C-74	50 oral	40-90	0	5 hr. +	Maximal return ectopic rate 15.
C-75	125 oral	180	0	5 hr.	Rigidity and involuntary movements for 1 hr. as effects reached max.
C-81	35 i.v.	60	0	4 hr.	After 4½ hr. ectopic rate rose to 90.
C-81	25 i.v.	90	0	Indef.	This dose 4½ hr. after last previous one (above). Ectopic rate rose to 25 after 2 hr. Max. return 40 next day.
C-85	250 oral	195	0	7 hr. +	Returned to max. ectopic rate of 50

i.p.—intraperitoneally; i.v.—intravenously; oral—via stomach tube; min.—minimal ectopic rate after administration of dilantin.

The maximal size of a single dose was 50 mg/kg. In all tests employing larger amounts multiple doses were given, the number varying up to 10.

morning (21-22½ hours) the ectopic rate varied between 140 and 170 per minute. Four doses of dilantin, 25 mg. per kg. each, given within a period of 2 hours at intervals of 20 to 53 minutes, eliminated all ectopic beats for 3 hours, and very few were recorded during the 9 hours of observation that followed their disappearance. During the next night low frequency (15-30/minute) ectopic ventricular beats returned.

The sinus rate usually declined also during the decline of ectopic frequency under the influence of dilantin. During the experiment illustrated in figure 3, prior to the

first dose of the test which began at 22½ hours, the sinus rate was 150 to 160 per minute. After ectopic complexes disappeared (25–26 hours) following dilantin the sinus rate was 105 to 110 per minute.

*Administration of Dilantin by Stomach Tube* produced results in some experiments that closely resemble those produced by the intravenous route except for a longer delay before effects were obtained. In one experiment, for example, dilantin, 125 mg. per kg., was given in 3 doses of 50, 50 and 25 mg. per kg. one-half hour apart via stomach tube. The test was begun 22 hours after occlusion with an ectopic rate (total heart rate) of 180 per minute. Within 15 minutes after the last of the 3 doses the ectopic rate reached zero and remained low (0–25) for about 3½ hours longer before a rising trend became evident. Reduction of the ectopic frequency lasted for more than 6 hours. During the period of low ectopic frequency the sinus rate showed a continuous diminution (except during a period of rigidity and spasmodic movements) which changed to a slow rise simultaneously with the rise in ectopic frequency.



Fig. 1. A, Control record made before operation; B, Ectopic ventricular tachycardia 10½ hours after occlusion and just before 1st dose of dilantin; C, Sinus rhythm in record taken before injection of 1st dose was completed.

In another experiment with dilantin by stomach tube, the test was begun with a totally ectopic rate of 180 per minute also. The drug was given in doses of 25 mg. per kg. at 30- to 60-minute intervals. The first evidence of diminution of ectopic activity was obtained, as before, after 125 mg. per kg. had been given. In this case the effect was less after this amount and developed more slowly. Doses were continued until a total of 250 mg. per kg. was given. The ectopic rate reached zero and the total rate 105 about 2 hours after the last dose (almost 8 hours after the first). About 3 hours later low-frequency ectopic beats returned again but never rose above 50 per minute. These 2 experiments are in agreement in that about 125 mg. per kg. were required before definite suppression of ectopic activity became evident. The slower development of effect and greater total amount of dilantin required for complete suppression in the latter experiment may be signs that absorption from the gastrointestinal tract was slower in that experiment.

*Penobarbital Sodium and Dilantin Sodium.* Tests were made to determine the effects of phenobarbital alone upon ectopic activity and also to observe the effects of dilantin in animals under the influence of phenobarbital but with persisting ectopic rhythms. The data are presented in table 2. In all experiments in which both drugs were administered, phenobarbital was given before dilantin and sufficient time was allowed after each dose to permit full development of its effect before another dose of either drug was given.

In 2 experiments (C-97 and C-98 in table 2) out of 8 in which phenobarbital in amounts of 30 mg. per kg. or more was given, the phenobarbital alone significantly reduced the ectopic rate, and no dilantin was used. In these 2 experiments, the ectopic rates were only moderately high (120-140 in one and 80-130 in the other) prior to medication. In each of these 2 experiments the ectopic frequency was reduced to 0 to 10/minute for about 30 minutes and reductions to one-half the rate before treatment or below were maintained for 2 hours in one case and  $1\frac{3}{4}$  hours in the other. In 6 experiments with higher ectopic frequencies, ranging from 175 to 240 prior to treatment, phenobarbital alone failed to produce complete suppression of ectopic impulses, though amounts up to 60 mg. per kg. were given. To eliminate ectopic activity dilantin was administered later. A chart to illustrate these results is reproduced in figure 4. Each dose of phenobarbital produced a reduction in ectopic rate

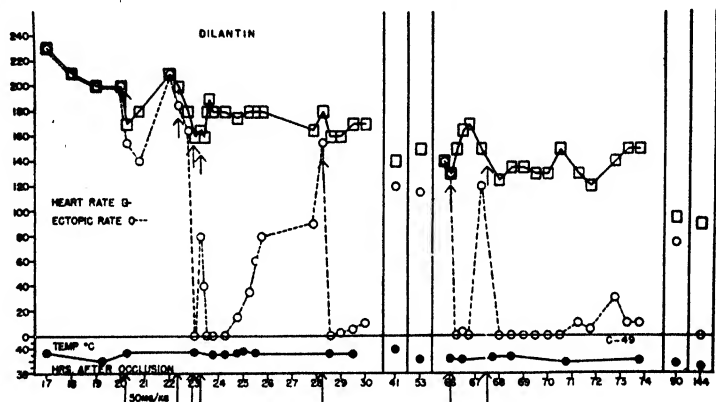


Fig. 2. EFFECTS OF DILANTIN on ectopic rhythms during 1st and 3rd post-occlusion days. Each arrow indicates the time of injection of dilantin 50 mg. per kg. Injection time 5 minutes each.

(the first 2 being large ones); however the reductions were of brief duration. Two doses of dilantin totalling 25 mg. per kg. eliminated all ectopic beats for more than 2 hours and the ectopic rate had risen to only 30 per minute 6 hours after the last dose. The prior and intercurrent administration of phenobarbital appears to have increased the effectiveness of dilantin to a marked degree.

A comparison of the data in tables 1 and 2 confirms the potentiation of effect. In experiments presenting a given high range of ectopic frequencies before testing, e.g. 180 per minute and above, the administration of phenobarbital greatly and consistently reduced the amount of dilantin required for prolonged suppression of ectopic activity. In 2 experiments (C-63 and C-65, table 2) as little phenobarbital as 10 mg. per kg. markedly enhanced the effectiveness of small doses of dilantin.

**Toxicity.** Two dogs were killed by rapid intravenous injection of dilantin. In both animals respiration ceased first and cardiac standstill followed shortly afterward. As little as 20 mg. per kg. proved fatal when injected quickly, but doses as large as 50 mg. per kg. were given repeatedly without alarming respiratory or cardiac changes during testing procedures in fully unanesthetized animals after the necessity

for slow administration was understood. Routinely, each dose was diluted to 20 cc. by addition of Locke's solution. This was administered slowly from a 20-cc. syringe. If 4 or 5 cc. of this dilution of a 50 mg. per kg. dose were given quickly the respiratory movements usually increased in rate and depth. This effect would subside after 10 to 20 seconds after which more of the dilantin solution could be given. The respiratory stimulation was repeated whenever administration became too rapid. It was regarded as a signal to wait, and to proceed more slowly. About 5 minutes was adopted as the minimal safe period for an injection. As a result of experience the respiratory changes were kept small in the later injections.

The administration of dilantin to a wide-awake dog usually produced restlessness. If the animal was asleep, or even under the influence of a small dose of barbiturate, the dilantin tended to awaken it. Mark-time walking or running movements sometimes were produced for periods as long as 10 to 30 minutes.

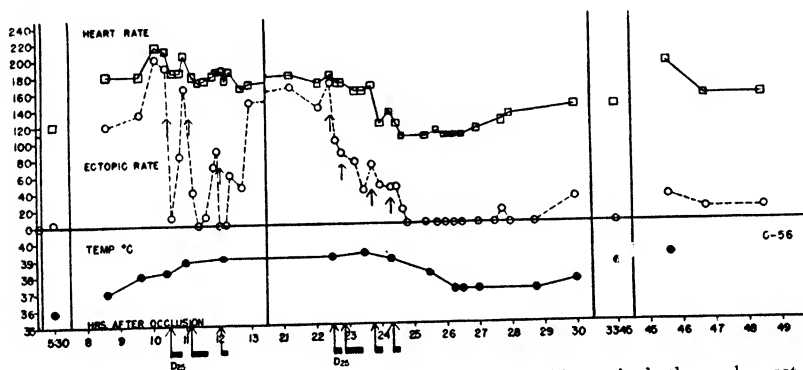


Fig. 3. EFFECTS OF DILANTIN soon after development of rapid ectopic rhythm and on 1st post-occlusion day. Each arrow indicates injection of dilantin, 25 mg. per kg. Length of flag on arrow indicates time taken for injection.

Rigidity of the limbs in a posture of extension with opisthotonus was observed in a few animals after the administration of large amounts of dilantin within a brief period. In some cases the extension was accompanied or followed by a period of coarse tremor or occasional jerking movements of the head or a limb. Such central nervous system symptoms were recorded in the protocols of 4 experiments. One of the periods of rigidity had a duration of about one hour (*exper. C-75, table 1*). Each of the others occurred soon after an injection and had a duration of only a few minutes. It is probable that these effects upon the central nervous system are similar to those which occur in patients receiving dilantin for anticonvulsant therapy (11).

Observations upon the deaths of 3 animals indicate that barbiturates increase the toxic effects of dilantin, though no systematic study of this relationship has been made. One animal was sacrificed under morphine-barbital sodium (180 mg/kg.) anesthesia by slow intravenous infusion of dilantin. The animal died after 150 mg. per kg. was infused in 1½ hours. Two other dogs were killed during tests begun 4 hours and 4½ hours after occlusion under pentobarbital sodium anesthesia. In one of these 2 animals, phenobarbital, 30 mg. per kg., followed by dilantin, 75 mg. per



kg., in 3 doses within one hour resulted in death. The other animal received phenobarbital, 60 mg. per kg., and dilantin, 45 mg. per kg., both in divided doses, before respiratory standstill supervened. It should be remembered that part of the effect of the anesthetic dose of pentobarbital sodium given for surgery still existed at the time these tests began.

TABLE 2. EFFECTS OF PHENOBARBITAL SODIUM AND DILANTIN SODIUM UPON VENTRICULAR ECTOPIC FREQUENCY

EXPER. NO.	DRUG	AMT., MG./KG.	ECTOPIC FREQUENCY		DURATION REDUC-TION TO $\frac{1}{2}$	REMARKS
			BEGINNING	MIN.		
C-63	Phenobarb.	10 i.v.	180	0	7½ hr. + indef.	Phenobarb. 10 and dil. 25 reduced ectopic freq. 2½ hr. Added dil. 10 eliminated them more than 5 hr. Max. return 55.
	Dilantin	35 i.v.				
C-65	Phenobarb.	10 i.v.	120	0	4½ hr. + indef.	Brief return to 70 after 4½ hr., then subsided to 0-10.
C-66	Phenobarb.	30 i.v.	180	0	7½ hr. +	7½ hr. after 1st suppression of ectopic beats, return to 75.
	Dilantin	36 i.v.				
C-76	Phenobarb.	10 i.v.	220	10	1 hr.	Sick animal. Pulmonary edema. Cardiac standstill 1 hr. 15 min. after last dose.
	Dilantin	60 oral				
C-77	Phenobarb.	30 i.p.	160	0	4 hr. +	Only reading above ½ of ectopic rate at 1st dose.
C-95	Phenobarb.	60 i.v.	175	0	5 hr. +	60 mg/kg. phenobarb. alone reduced ectopic freq. to 40-90.
	Dilantin	25 i.v.				
C-96	Phenobarb.	30 i.v.	100	0	2½ hr.	30 mg/kg. phenobarb. alone did not reduce ectopic rate.
C-97	Dilantin	20 i.v.				Low ectopic rate interrupted by paroxysm 185-190 2 hr. after dose. Returned after 15 min. to 0-75.
	Phenobarb.	30 i.v.	120-140	0	2 hr. +	
C-98	Phenobarb.	30 i.v.	80-130	0	1½ hr.	Return began after 40 min.
C-99	Phenobarb.	50 i.v.	220	0	5 hr. +	Last record ectopic rate 10. Sacrifice.
	Dilantin	60 i.v.				
C-100	Phenobarb.	65 i.v.	180-240	0	8 hr. +	Phenobarb. effect brief. Ectopic rate 70-80 next day.
	Dilantin	25 i.v.				

Maximal single dose of phenobarbital, 30 mg/kg.; dilantin 50 mg/kg.  
i.v.—intravenously; oral—via stomach tube.

The amount of dilantin (given slowly) which a dog can tolerate in the absence of other drugs is not adequately known. Scherf's (12) 4 dogs were anesthetized by excessive doses of pentobarbital sodium (50 mg/kg.). They died after slow administration of 66 to 69 mg. per kg. of dilantin. Our experience indicates that the unanesthetized dog can tolerate more than three times these amounts within 3 or 4 hours. One dog received 150 mg. per kg. in less than one hour without exhibiting toxic symptoms. Each of these dogs had the added stress of a large acute myocardial

infarct and rapid ventricular tachycardia. There were no fatalities among the animals that received large amounts (100–250 mg/kg.) during the course of a test. Gruber and coworkers (13) who studied toxic actions of dilantin did not determine lethal doses in dogs and the factor of anesthesia seems to have been ignored in estimating toxic effects.

The administration of phenobarbital before dilantin in our experiments rendered unnecessary the use of large amounts of dilantin to suppress ectopic ventricular activity. The only toxic reaction noted in the dogs receiving both drugs after full recovery from the surgical anesthetic before testing began was vomiting. This occurred on 3 occasions immediately after the administration of dilantin following

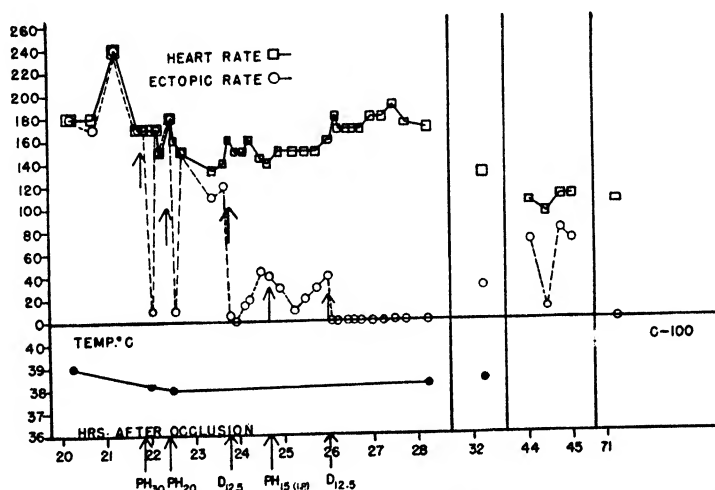


Fig. 4. EFFECTS OF PHENOBARBITAL AND DILANTIN. PH<sub>30</sub> = phenobarbital, 30 mg. per kg. D<sub>12.5</sub> = dilantin, 12.5 mg. per kg.

phenobarbital. On each occasion a single stomach-emptying ejection completed the process. There was no subsequent retching.

During the trials when dilantin was used alone in unanesthetized dogs, vomiting occurred only once. In that case the animal had finished eating 350 gm. of dog meal and milk a short time before the injection. Vomiting may be regarded as a rare occurrence following the injection of dilantin into dogs that are not under the influence of barbiturate.

**Blood Pressure.** Arterial pressures were measured in anesthetized animals only. Intravenous injection of dilantin, 12.5 mg. per kg., in one minute promptly reduced mean blood pressure from 90 to 55 mm. Hg. This was followed by rapid recovery. Within 2 minutes the mean pressure rose to 80 mm., and had returned to the control reading of 90 mm. at the end of 3 minutes. An identical dose given to the same dog 2 hours later was injected more slowly, the injection being given in 4½ minutes instead of one. The mean arterial pressure decreased from 110 to 100. With the faster injection and large decrease in mean pressure there was a great increase in the rate

and depth of respiration, but with slow administration and a small decline in mean pressure the respiratory change was hardly detectable. It is probable that the increases in respiratory movements described earlier as being observed during injections of dilantin were due to reflexes resulting from diminution in arterial pressure. In each of the trials cited, with both large and small changes in blood pressure the disappearance of ectopic complexes from the record far outlasted the fleeting diminution in blood pressure.

#### DISCUSSION

The findings in these experiments demonstrate that dilantin in adequate dosage produces cessation of ectopic impulses in a ventricle undergoing myocardial necrosis, and that phenobarbital enhances this action. These effects of dilantin and of phenobarbital appear to be analogous to their effects upon spike discharges in the brain in focal epilepsy and may be regarded as presumptive evidence in support of the view that the excitatory processes or substances which initiate the discharge of impulses in the boundary of a myocardial infarct may be similar in some fundamental property to the excitatory factors which produce epileptogenic spike discharges in boundary zones about post-traumatic scars and certain other cerebral lesions (8-10).

Changes in the excitability and automaticity of cardiac tissues which result from a variety of chemical, electrical and temperature alterations in cellular environment have been shown to be markedly similar to the changes produced in excitability and automaticity of nerve and skeletal muscle by similar environmental changes. Anoxia increases excitability in nerve and in cardiac muscle soon after deprivation of oxygen, or if the degree of oxygen lack is moderate, and depresses excitability in both if the anoxia becomes severe (7,14). Increased concentration of calcium ions diminishes excitability in turtle auricle and ventricle (15), dog Purkinje fibers (16), nerve (17), skeletal muscle and motor end plates (18). Addition of calcium ions to fluid bathing spontaneously discharging mammalian left auricle (19), Purkinje fibers (19), nerve (17), or skeletal muscle (18) causes diminution or cessation of the spontaneous activity. Reduction of calcium ions in the medium induces automaticity in skeletal muscle (18), nerve (17), and presumably in Purkinje fibers since the reverse reaction occurs. Veratrin increases negative after-potential and repetitive discharges in nerve (20,21), Purkinje fibers (22), and skeletal muscle (23) after an initiating stimulus. Local cooling facilitates the excitation of nerve fibers at artificial synapses (24) and the initiation of repetitive discharges in mammalian ventricles following the arrival of an impulse (25).

This list of similarities of reactions of heart and other tissues does not mean that complete correspondence can be demonstrated. An apparent inconsistency is found in the production of ectopic beats by injecting calcium salts into the circulation of turtles (15) and dogs (26). These discharges may be due to facilitation of excitation at sympathetic nerve terminals by calcium ions (27,28), or they may result from an increase in supernormality (29) which more than balances the depression of excitability that exists after the recovery process is complete. Repetitive responses to excess calcium ions have not been reported in tissues other than heart muscle, however. Repetitiveness and decreased resting excitability coexist in certain nerves treated with veratrine (21).

The property of recovery through supernormality explains the production of an ectopic beat following a normal one in cardiac bigeminal rhythms and the production of trains of discharges following an initiating stimulus in various heart, skeletal muscle and nerve tissues (29,21) under a variety of conditions. However recovery through supernormality does not seem to be a characteristic feature of acute myocardial infarction since coupling is not seen often in coronary occlusion experiments.

Rate of accommodation is an important factor in automaticity. A low rate of accommodation facilitates repetitive activity; a high rate of accommodation opposes it (30). Anodal polarization reduces accommodation and favors repetition in nerve (31); it produces accelerating ectopic rhythms and ventricular fibrillation if applied to mammalian ventricles with an intensity slightly above the anodal threshold for ectopic discharges (32).

Low rate of accommodation, increased excitability and increased supernormality during recovery are the known properties which favor automaticity. Since evidence of supernormality usually is lacking in experimental myocardial infarction it appears probable that low rate of accommodation and increased excitability (1) are the principal factors in the initiation of ectopic ventricular tachycardia in these hearts.

It probably is significant that dilantin which abolishes ectopic ventricular discharges following coronary occlusion also abolishes repetitive responses in frog nerves which respond repetitively without special treatment or that have been made repetitive by soaking in phosphate solution (33). It may be inferred that this treatment lowers the rate of accommodation by de-ionizing calcium and that dilantin abolishes the repetitiveness in the nerve by increasing accommodation. Little change in the threshold to induction shocks was reported.

Since dilantin and phenobarbital have proved to be effective in suppressing the discharge of ectopic impulses in acute myocardial infarction, other drugs with anti-epileptic action should be tested in these cardiac preparations. These findings place a new emphasis upon the concept of unity of fundamental mechanisms of excitation.

A corollary to these results is the suggestion that the dog with ectopic ventricular tachycardia may be useful in the search for better anti-epileptic drugs.

#### SUMMARY

The concept that the mechanism of production of ectopic ventricular impulses in acute myocardial infarction may be due in part to factors that are similar to those which evoke focal cerebral impulses that produce epileptic seizures led to the trial of anti-epileptic drugs for suppressor effect upon ventricular ectopic activity. Diphenylhydantoin sodium (dilantin) in sufficient dosage produced complete cessation of ectopic activity for a period of time followed by a longer period of marked reduction of ectopic frequency. The quantity of dilantin required was greater when the ectopic frequency was greater and less when the ectopic frequency was less. Dilantin reduced the frequency of discharge of the S-A node also, but the effect upon the frequency of ectopic discharges was much greater. Phenobarbital sodium alone was effective in suppressing only relatively low-frequency ectopic rhythms. It markedly increased the effectiveness (reduced the required dose) of dilantin in the suppression of ectopic rhythms of all frequencies. Rapid intravenous administration of dilantin

produced a sharp drop in blood pressure and an increase in rate and depth of respiration. In 2 trials these phases were followed by cessation of respiration followed by cardiac standstill. Slow intravenous administration produced little or no change in blood pressure or respiration. In a few trials the administration of sufficient dilantin alone to suppress all ectopic activity produced toxic nervous symptoms; after phenobarbital, the relatively small amounts of dilantin required never produced such symptoms.

In the discussion an incomplete listing of similarities of reactions of cardiac muscle, nerve and skeletal muscle is given. It is suggested that ventricular preparations with ectopic rhythms might be valuable for preliminary testing of drugs for anti-epileptic activity.

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COMPARISON IN DOGS OF PLASMA VOLUME  
MEASURED WITH T-1824 AND WITH ANTIGENS<sup>1,2</sup>

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THE dye-method (or any similar method) for measuring plasma volume poses two questions which still remain controversial in spite of having received a good deal of attention from investigators: At what time after the injection of dye (or other suitable test substance) is mixing complete? How much of the injected dye escapes during the period of mixing? Of recent years most of the workers who have employed the blue dye T-1824 for measuring plasma volume have proceeded on the assumption that the contour of initial steeper portion of the time-concentration curve is determined by mixing and that the subsequent portion, which is linear on a semilog plot, represents disappearance of dye. On the basis of this interpretation, extrapolation of the disappearance curve to the time of injection is assumed to correct for the dye lost during mixing. If, however, a considerable amount of dye is lost initially by rapid staining and phagocytosis, as some have claimed (1), then the above extrapolation procedure is certainly not valid and the estimated plasma volume must be too high.

Additional interest now attaches to this problem because of the considerable evidence published in recent years showing that the cell-tagging methods yield a smaller value for total blood volume than does the dye method (2-4). If a significant fraction of the injected dye is regularly 'gobbled up' during the mixing period one would have an easy explanation for the discrepancy. If not, then the solution must be sought elsewhere.

The experiments reported here were designed to test for loss of the dye T-1824 during the mixing period by comparing directly the plasma volume measured with the dye and with three antigens, bovine albumin, bovine globulin and the polysaccharide SIII. If T-1824 is to any considerable extent removed by staining and/or phagocytosis during the mixing period, the plasma volume calculated from the usual extrapolation of the dye curve should be greater than that given by the simultaneous determination with a known amount of one of the antigens.

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## PROCEDURES AND METHODS

The tests were made on 16 mongrel dogs ranging in weight from 7 to 13 kg. Four were splenectomized. Ten experiments were done with T-1824 and bovine albumin, two with T-1824 and bovine globulin, and four with T-1824 and the polysaccharide SIII. In each instance, measured amounts of the dye and the antigen were pipetted into a syringe and injected together. The precautions observed and general procedure for drawing and handling blood samples and for making the injections have been described elsewhere (5-7) and only a few details need be mentioned here. The dye and antigen determinations were made on serum from blood collected in oiled tubes. Dye concentration was determined with a Koenig-Martens visual spectrophotometer. Not less than 8 to 10 blood samples, conveniently spaced over a period of 120 minutes after the injection were collected in order to have sufficiently well-defined time-concentration curves for accurate extrapolation. Fluid shifts during a test (indicated by fluctuations in the plasma protein concentration or the hematocrit value) were rarely large enough to call for a correction (7) of the observed dye or antigen concentration. The extrapolations for estimating the theoretical concentration,  $C_0$ , of dye or antigen at zero time, were all done on semilog plots (8). The plasma volume was then calculated from  $C_0$ , according to an appropriate modification of the simple formula  $V_0 = \frac{C_1 \times V_1}{C_0}$  where  $C_1$  and  $V_1$  stand for concentration and volume of the standard dye or antigen injected, and  $V_0$  is the plasma volume.

The T-1824 used throughout the investigation was a standard aqueous solution in ampules prepared by Warner Institute, New York (7). Diluted 1:250 in dog serum and read at 624 m $\mu$  in the Koenig-Martens spectrophotometer the optical density (1-cm. cuvette) of this dye lot (no. 3) was 1.63.

*Antigens.* The bovine albumin used was lot ACB11, Armour Laboratories. The bovine globulin was Fraction II, Armour Laboratories.<sup>3</sup> The polysaccharide from pneumococcus type SIII (referred to hereafter as SIII) was provided by Dr. Michael Heidelberger.<sup>4</sup> The antisera were obtained from rabbits treated with the above antigen preparations.

*Serological titration of the antigen.* The reagents needed for the precise titration of the soluble antigen include: 1) the antigen itself, which may be some relatively pure protein foreign to the experimental animal such as bovine serum albumin or fraction II of bovine globulin, or it may be a nonprotein antigen such as type III soluble specific polysaccharide, 2) specific precipitating antisera for each antigen used, obtained by the repeated injection of the corresponding antigen into rabbits, 3) sterile buffered saline at pH of about 7.0 and, in experiments such as those described here where the foreign antigen is to be measured in dog's serum or plasma, and 4) a pre-injection control sample of the dog's serum or plasma.

With these reagents an accurate titration of unknown amounts of antigen in the dog's blood is possible by means of the photoreflexometer (9), an instrument designed for the measurement of turbid systems. Titrations of known and unknown

<sup>3</sup> Both proteins were kindly provided by Dr. Edwin Cohn, Harvard Medical School.

<sup>4</sup> We wish to thank Dr. Heidelberger for the SIII as well as for the corresponding antiserum (B65RS) used in the titrations and for his generous advice and interest in the determination of SIII.

amounts of the antigen SIII have been made by Libby (9), by Bukantz, Cooper and Bullowa (10) and Martin (11). The titration is based on the proportionality between the amount of turbidity and the amount of antigen reacting with constant amounts of antiserum, in the region of relative antibody excess. Thus a standard titration curve is first prepared using a particular antiserum and titrating it with the corresponding antigen in buffered saline plus amounts of control dog serum comparable to those used in an actual determination of blood volume. A standard curve obtained from the titration of an antigen in buffered saline only cannot be used as it may lead to errors. Thus a given dilution of antigen in the region of antibody excess may readily give 10 per cent greater turbidity if 2 per cent dog serum or plasma is present in the antigen dilution, than when the antigen is diluted with saline alone. This is probably due to the complement present in the dog's serum, for it has been shown that complement has a considerable effect on the quantitative titration of precipitating antisera (12, 13). Also, it was found that inactivation of the normal dog serum used in these experiments reduces this effect. In the titrations it is not necessary that the absolute amounts of antigen in the standard solution be known, provided the same standard solution of antigen used for tests on the dog's blood *in vitro* or *in vivo* form the basis for the antigen dilution in the standard curve.

The final volume of the sample to be read in the photronreflectometer had to be 2 ml. to bring the level of fluid above the cell window. Constant amounts of antiserum (0.3 ml.) were added to constant amounts (1.7 ml.) of the antigen in the dilution needed. Before the addition of antiserum a control reading is made for each dilution of antigen used, and this amount of galvanometric deflection is subtracted from the total amount of deflection resulting from the addition of the antiserum, after a 20-minute incubation period in an air incubator at 32.5°C. An antiserum control value is also obtained from the incubation of 0.3 ml. of antiserum in saline. Full details of the technique of titration by means of the photronreflectometer may be found in the accounts by Boyden (14) and Boyden and De Falco (15).

*In vitro determination of plasma volume with T-1824 and antigen.* The titration curve for bovine albumin is shown in figure 1. This was obtained from titrating antiserum no. 117 (anti-albumin serum obtained from a rabbit) with various dilutions of the standard 5 per cent bovine albumin solution made up in buffered saline containing 2 per cent plasma (1 part in 50) from dog 857 at the 2500 level of antigen dilution. All antigen dilutions were made directly from the standard 5 per cent albumin solution, this standard solution being the same material which was later added to the dog's blood for the *in vitro* determination of plasma volume with the antigen.

The *in vitro* test was made on 50 ml. of heparinized blood (dog 857). After addition of exactly 0.195 ml. of the standard T-1824 solution and 0.5 ml. of the standard 5 per cent bovine albumin the blood was agitated gently for several minutes to insure thorough mixing. Several samples were then centrifuged in 4 cc. hematocrit tubes (3000 rpm. for 30 minutes, radius 15 cm.) to determine the hematocrit value and the plasma fractions used for the serological and spectrophotometric analyses.

For the antigen determinations, 0.5 ml. samples of the plasma (containing dye and albumin) were measured into 25 ml. flasks and the latter then filled to the mark with buffered saline. These diluted unknowns thus contained 2 per cent dog's plasma,



the same dilution of dog plasma used in the preparation of the standard curve of reference.

Four sets of triplicate readings were made on 0.5 ml. samples withdrawn from the unknown. When diluted 1:50 during the titration with the antiserum they gave galvanometric readings (empirical turbidities) as follows:

a	b	c	d
43.4	43.8	43.0	42.6
43.1	42.4	43.2	43.2
43.2	43.1	43.1	42.8
—	—	—	—
Av. 43.1	43.1	43.1	42.9

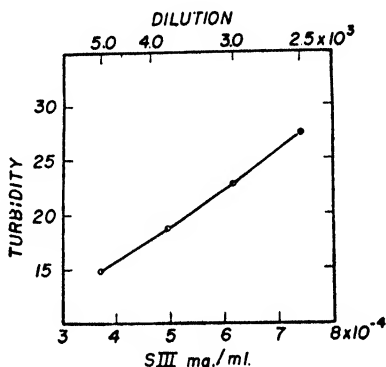
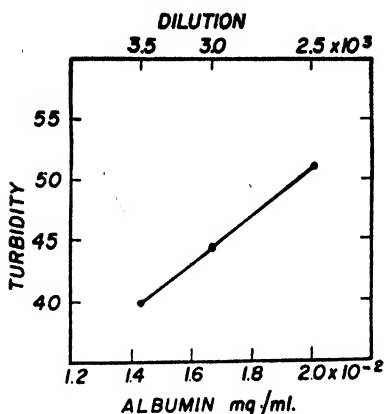


Fig. 1 (left). STANDARD TITRATION CURVE of bovine albumin ACB 11 in buffered saline plus 2 per cent dog's serum. Concentration of albumin is plotted below and dilution of albumin is shown above as so many times the original standard (5 %). Turbidity readings are given along the ordinate.

Fig. 2 (right). STANDARD TITRATION CURVE of SIII in buffered saline plus 2 per cent dog's serum (plotted as in fig. 1). Standard solution of SIII contained 1.85 mg/ml.

The grand average of the 12 readings was 43.1. From the titration curve in figure 1 it will be seen that this turbidity corresponds to an antigen concentration of .00160 mg/ml. or a dilution of 3120 times. Since the unknown sample was diluted 50 times during titration and the total dilution was 3120 times, the remaining dilution  $3120/50 = 62.4$  times, was brought about by adding 0.5 ml. bovine albumin to the 50 ml. of blood. In order to dilute the 0.5 ml. of bovine albumin 62.4 times a volume of plasma of 31.2 ml. would be necessary. The formula for calculating the plasma volume can therefore be stated as follows:

$$\text{plasma volume in cc.} = \frac{\text{total dilution (as read from the turbidity)}}{\text{dilution in preparation of the unknown}} \times \text{ml. of antigen added}$$

or

$$\text{plasma volume in cc.} = \frac{50 \text{ mg/ml.} \times 0.5 \text{ ml.}}{.00160 \text{ mg/ml.} \times 50}$$

In this experiment the plasma volume determined with T-1824 was 31.3 cc. The results show as much agreement between the dye and antigen determinations as can be expected. The plasma volume calculated from the average centrifuge hematocrit value (39.5%<sup>6</sup>) and from the total volume in the flask (50.7 ml.) was 30.7 cc.

The titration curve for SIII prepared in the same manner as described for bovine albumin, is shown in figure 2. The standard solution of SIII contained 1.85 mg/ml. The *in vitro* tests with T-1824 and SIII were done on heparinized blood from dog 857. Three flasks were prepared, each containing 50 ml. of blood, 0.5 ml. of the standard SIII solution and 0.195 ml. of the standard T-1824 solution. After mixing, six 4-cc. samples were taken from each flask and centrifuged to obtain the hematocrit value. The plasma fractions were analyzed for dye and antigen.

The SIII titration of 3 separate samples from each of the 3 flasks gave titration readings as follows:

Flask 1	Flask 2	Flask 3
22.9	22.6	23.3
22.1	24.0	23.2
23.8	23.4	23.4
—	—	—
Av. 22.9	23.3	23.3

By referring to the titration curve in figure 2, these turbidity readings may be expressed in terms of dilution or as concentration of SIII, whichever is preferred. If concentration is used in the calculation of volume we have for *Flask 1* the following:

$$\text{plasma volume} = \frac{1.85 \text{ mg/ml.} \times 0.5 \text{ ml.}}{.000167 \text{ mg/ml.} \times 50} = 30.0 \text{ ml.}$$

The plasma volume determined with T-1824 was 29.8 ml.

A summary of the results of 4 beaker experiments comparing the antigen and dye measurements of plasma volume is presented in table 1. Included also are the centrifuge hematocrit values for comparison with the relative cell volumes calculated from the dye and antigen determinations. The data in table 1 demonstrate that the antigen concentration in blood can be satisfactorily determined by the titration method provided the turbidity readings on the unknown samples are referred to a standard titration curve prepared with control plasma from the same animal.

## RESULTS

We are dealing here with test substances which all escape rather slowly from the blood stream. In comparison with the amount so lost, the portion removed by taking samples may be significant. This factor might or might not influence the apparent disappearance rate depending on whether or not the animal compensates for the volume of plasma removed in the samples. If the plasma volume is maintained by influx of tissue fluids, the slope of the disappearance curve will be increased to an extent determined by the fraction of the total volume that is withdrawn in samples. The volume of each sample is routinely recorded on the standard protocol sheet used in this laboratory. From this information and from the hematocrit value, together

<sup>6</sup> Includes buffy coat.

with the data available in the time-concentration curve, the latter can be adjusted for the amount of test substance lost through sampling. In the present series of experiments the volume removed in the course of 2 hours was of the order of 5 per cent of the blood volume. This is sufficient to make a substantial difference in the estimation of the disappearance rate.

An example of applying the correction for sampling is shown in figure 3 (*dog 6*). According to the original curves indicated by solid lines, the losses of T-1824 and bovine albumin during the first hour were 12 and 9 per cent respectively; after ad-

TABLE 1. IN VITRO DETERMINATIONS OF PLASMA VOLUME WITH T-1824, BOVINE ALBUMIN AND SIII<sup>1</sup>

	PLASMA VOLUME		RELATIVE CELL VOLUME			PLASMA TRAPPING	
	T-1824	Albumin	T-1824 (calc.)	Albumin	Centrifuge <sup>2</sup> (observed)	Dye	Albumin
	ml.	ml.	%	%	%	%	%
I	31.3	31.2	38.3	38.5	39.5	3.0	2.5
		SIII		SIII (calc.)			SIII
II	29.8	30.0	41.2	40.8	43.1	4.4	5.3
III	29.7	29.5	41.4	41.8	43.1	3.9	3.0
IV	29.7	29.6	41.4	41.6	43.0	3.7	3.3
						av. 3.8	3.5

<sup>1</sup> Tests were made on 50-ml. samples of heparinized dog's blood. To each sample was added 0.195 ml. of the standard T-1824 and 0.5 ml. of the standard antigen solution giving a final volume of 50.7 ml. Each value for plasma volume in this table is average of triplicate determinations. Each value for relative cell volume as determined by centrifugation (30 minutes, 3000 rpm, radius 15 cm.) is average of 6 observations. Values for relative cell volume listed under T-1824, SIII and albumin (true relative cell volume) are calculated from total volume and plasma volume as follows:

$$\text{Relative cell volume} = \frac{50.7 \text{ ml.} - \text{plasma volume ml.} \times 100}{50.7 \text{ ml.}}$$

Plasma trapped in the cell mass after centrifugation is given here as percentage of centrifuge volume. It is calculated as follows:

$$\text{Per cent trapping} = \frac{R. C. V_{\text{centrifuge}} - R. C. V_{\text{true}}}{R. C. V_{\text{centrifuge}}} \times 100$$

<sup>2</sup> Includes buffy coat.

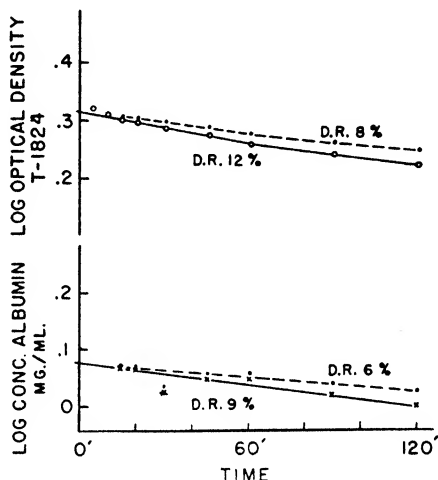
justing for the amounts of T-1824 and albumin removed in the samples the values were 8 and 6 per cent respectively. The adjustment thus made in the time-concentration curves to estimate the true disappearance rate does not, however, alter the level at which the disappearance curve intercepts the zero time ordinate. The plasma volume is therefore the same whether calculated from the original or from the corrected curve.

The results from 16 experiments in which plasma volumes were measured simultaneously with T-1824 and one of the antigens are listed in table 2 in the order in which the tests were carried out. Only one determination with antigen was made on each dog in order to avoid the possible complications from sensitization. Nevertheless, 2 animals, 5 and 13, showed signs of circulatory distress within 15 to 20 minutes after

the injection of the antigen, in the first instance bovine albumin, and in the second SIII. The veins collapsed, samples were obtained with some difficulty and the blood was visibly darker in color. The heart rate became rapid and the respiration faster. In both animals the effects lasted one-half to three-quarters of an hour. The period of circulatory disturbance was accompanied by fluctuations in the hematocrit values and plasma protein concentration. In *dog 13* this took the form of a temporary and quite definite dip in both the dye and SIII time-concentration curves.

**Plasma Volumes.** The results in table 2 give no indication that the plasma volume measured with antigens is essentially different from that measured with T-1824. The differences in volume, expressed as percentage of the dye volume that the antigen volume is above or below the dye volume, range from +8.4 to -5.8 per cent with 7 experiments on the plus and 9 on the minus side. In 10 experiments with bovine

Fig. 3. SIMULTANEOUSLY DETERMINED TIME-CONCENTRATION curves for T-1824 and bovine albumin with log of optical density of T-1824 and log of concentration of bovine albumin in mg./ml. plotted against time in minutes after injection of 2 cc. of 0.46 per cent T-1824 and 10 cc. of 5 per cent ACB 11. *Dog 6*, splenectomized, body weight 7.3 kg. Solid lines represent the original uncorrected curves; dotted lines represent curves which have been corrected for amount of dye or albumin removed in blood samples. It will be noted that adjustment for sampling does not alter value of  $C_0$  estimated by extrapolation of disappearance curve to time of injection.



albumin the average plasma volume was found to be 553.7 ml., as against 549.5 ml. with T-1824. In 2 experiments, 11 and 12, bovine globulin gave an average volume of 630.5 ml., the dye 618 ml. In 4 experiments the average value with SIII was 500 ml., with T-1824, 508 ml. The average from all 16 tests was 549.9 ml. with antigen and 547.7 ml. with T-1824.

In figure 4 the individual dye-volumes have been plotted against the antigen volumes. The linear correlation coefficient for these data was found to be .954.

**Disappearance Rates.** The values estimated from the uncorrected and corrected curves are shown in table 2 under columns designated *A* (apparent disappearance rate), and *B* (true disappearance rate), respectively. It may be noted that the disappearance rates of T-1824 were unusually high in *dogs 4* and *5*. Without implication that it constitutes an explanation, the fact should be mentioned that by the time the standard titration curve had been prepared on *dog 4* it was observed that this animal was in the initial stages of distemper. The test was nevertheless carried out. As for *dog 5*, it was noted above that this animal was one of the two that displayed a

'foreign protein reaction.' With these two exceptions the disappearance rates of T-1824 are for the most part in the range commonly encountered in normal dogs.

TABLE 2. PLASMA VOLUMES MEASURED SIMULTANEOUSLY WITH T-1824 AND WITH ANTIGENS IN 16 DOGS<sup>1</sup>

Dog No.	PLASMA VOLUME						DISAPPEARANCE RATE			
	Body wt.	Hct.	P. P. <sup>2</sup>	T-1824	Albumin	Difference	A (Apparent)		B (True)	
	kg.	%	%	ml.	ml.	%	T-1824 % hr.	Albumin % hr.	T-1824 % hr.	Albumin % hr.
1	13.2	44.9	5.5	652	680	+4.3	9	7	7	4
2	11.7	48.4	5.9	580	600	+3.5	15	12	12	9
3	9.6	53.7	5.9	472	460	-2.5	13	4	11	1
4	12.4	46.0		620	591	-4.7	23	7	17	3
5	12.2	42.3	6.1	682	656	-3.8	22	15	19	12
6 <sup>3</sup>	7.3	43.4	5.9	401	413	+3.0	12	9	8	6
7	8.1	50.0	6.7	484	500	+3.3	8	3	6	1
8	7.8	40.8	6.8	502	486	-3.2	6	3	4	1
9	10.0	42.1	7.0	552	555	+0.5	6	4	4	2
10 <sup>3</sup>	9.0	36.0	5.5	550	596	+8.4	8	4	5	2
				Av. 549.5	553.7					
					Globulin			Globulin		Globulin
11	9.6	44.2	5.9	684	671	-1.9	9	4	7	3
12	10.7	55.1	6.6	552	590	+6.9	11	6	8	5
				Av. 618	630.5					
				SIH	SIH			SIH		SIH
13	10.1	38.6	5.9	552	520	-5.8	10	4	8	2
14	10.8	42.8	6.9	488	486	-0.4	9	3	7	0
15 <sup>3</sup>	8.7	43.0	6.3	513	532	+3.7	12	0	10	0
16 <sup>3</sup>	9.7	41.8	5.8	479	462	-3.5	13	1	10	0
Av.	10.06	44.54	6.18	508	500					
Av. of all				547.7	549.9					
Corr. Coeff. = .954										

<sup>1</sup> Volumes are calculated from  $C_0$  by extrapolation of time-concentration curve on a semilog plot.

Disappearance rate is defined as follows:  $D.R. \% / \text{hr.} = \frac{C_0 - C_{50 \text{ min.}}}{C_0} \times 100$

Disappearance rates under columns designated A were derived from original time-concentration curves, uncorrected for amount of dye or antigen removed in blood samples. Values under B, from time-concentration curves corrected for sampling. For discussion see text.

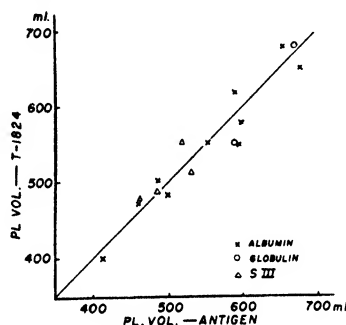
<sup>2</sup> From refractive index of serum. <sup>3</sup> Splenectomized.

Although it must be emphasized that the number of experiments is too small and the variability in the data too great to reach any definite quantitative values for the relative diffusibility of the antigens, the results indicate that the ratio of antigen/

dye lost is as one might expect smallest for the SIII. Globulin with a molecular weight twice that of albumin should escape more slowly than the latter and perhaps this could be demonstrated in a more exhaustive study. The antigen SIII, which tends to form large aggregates, and therefore in all probability has a larger effective molecular size than either bovine albumin or globulin appears to be lost so slowly that the correction for the amount lost in samples practically speaking accounts for the observed decrease in concentration during the first 2 hours after injection.

The amount of antigen remaining in the blood stream after longer periods of time was tested in only 4 experiments, 6, 7, 11 and 12. In *experiments* 6 and 7 with bovine albumin, the plasma levels after 24 hours were 38 and 57 per cent of the initial concentration respectively. In *experiments* 11 and 12 with bovine globulin, the 24-hour plasma levels were 52 and 51 per cent respectively. A 48-hour sample was also taken in *experiment* 12 showing a level of 38 per cent of the initial concentration.

Fig. 4. PLASMA VOLUME as determined with T-1824 plotted against volume simultaneously measured with antigen. Linear correlation coefficient is .954.



#### DISCUSSION

We have here results that have a bearing on 2 questions of importance in the determination of blood volume with T-1824: *a*) the magnitude of the error in estimating the true relative cell volume from the hematocrit, and *b*) the question as to whether or not the estimate of the plasma volume with T-1824 is too large because of an initial rapid loss of dye during the mixing period.

It may be seen from the results of the *in vitro* tests on known volumes of heparinized blood, summarized in table 1, that the relative cell volumes calculated from the dye and antigen determinations are identical within experimental error. The difference between these values and those obtained by the hematocrit method, attributable to plasma trapping, ranges from 2.5 to 5.3 per cent, the average for T-1824 being 3.8 per cent and for the antigens 3.5 per cent. These results are in line with those reported several years ago by Gregersen and Shiro (16) who used both T-1824 and Brilliant Vital Red, and also agree with the findings of Shohl and Hunter (17) and of Barnes, Loutit and Reeve (18) who have taken the trouble to re-examine the extent of plasma trapping rather critically. On the basis of our published results and periodic checks, we have for several years applied a factor of 0.96 (7) to the hematocrit value obtained by centrifugation for 30 minutes at  $1500 \times g$ . Reeve and his co-workers (18) using the same force and duration of centrifugation have chosen a factor of 0.95. Better

agreement in the results from widely separated laboratories could scarcely be expected.

The outstanding exception seems to be the report by Chapin and Ross (19) claiming 8 to 9 per cent plasma trapping. Careful perusal of their report and discussions with one of the authors have not uncovered a satisfactory explanation for the striking difference between their findings and those of others. The difference is the more remarkable since Chapin and Ross claim to have centrifuged their hematocrit samples longer and at higher centrifugal force (one hour at  $1800 \times g$ ) than used by either Barnes *et al.* (18) or by ourselves. Loutit, Barnes and Reeve (18) have suggested that in the preparation of dye standards inadequate time may have been allowed for combination of dye with protein. This is not very likely in view of the time ordinarily required for preparation of a standard, mixing the contents and completing a set of photometric readings, and the fact that 8 solutions of different strengths were prepared by Chapin and Ross in determining the standard K value for T-1824 in plasma. That this could be the source of a systematic error must furthermore be discounted if one accepts the results of one experiment in which plasma trapping determined by plasma protein dilution was also found to be over 8 per cent. However explicitly the conditions of centrifugation have been defined by Chapin and Ross one is led to conclude that some factor must have been present which consistently interfered with packing of the red cell mass.

The second question as to whether or not T-1824 measures too large a plasma volume because of rapid initial loss of the dye is answered by the results presented in table 2 and figure 4. These tests demonstrate that within limits of experimental error the volume distribution of T-1824 is the same as that of the three antigens.

The agreement in plasma volumes measured with four such different test substances could scarcely be accidental. Bovine albumin and bovine globulin, both proteins, have molecular weights of 68 to 70,000 and 150 to 160,000, respectively, and SIII, a polysaccharide which tends to form large aggregates probably has a molecular size equivalent to a molecular weight of several hundred thousand. Although T-1824 has a molecular weight of only 960 it too escapes slowly from the blood stream because its movement is restricted by the plasma albumin to which it becomes bound (8, 20, 21). Although the T-1824-albumin bond is reversible the energy of binding is so large that in the concentrations ordinarily employed for measuring plasma volume practically all of the circulating dye (999 parts in 1000) is in the bound form (21). Furthermore, Allen and Orahovats (22) have shown that the dye reacts so much more readily with albumin than with tissue cells that the probability of its being dislodged or adsorbed from the circulating albumin in significant amounts of staining or phagocytosis is extremely small, as indeed one would conclude from the slow disappearance rate itself. In short, T-1824 in the circulating blood can be considered essentially equivalent to tagged albumin. Hence one would expect the volume of distribution of the dye to be identical with that of bovine albumin, a substance of similar molecular size. Provided one corrects for loss of the test substance during the mixing period there is evidently no advantage in using a much larger molecule such as SIII although its disappearance rate is perceptibly less than that of either T-1824 or albumin.

Recent tests with plasma proteins tagged with radioactive iodine do not reveal

that these are superior to T-1824 for measuring plasma volume (23). The rates of disappearance are of the same order of magnitude and the volume of distribution seems to be the same.

The present evidence rather definitely disposes of the claims made by Cruickshank and Whitfield (1). As a matter of fact their interpretations of the T-1824 time-concentration curves were based on erroneous criteria of complete mixing and the changes in the mixing curves which they ascribed to the reticulo-endothelial system could easily be the result of circulatory changes which were not even considered in their experiments. Pickering and Dow (24) have recently published an instructive paper dealing with this question. Furthermore, the claim that the first injection of dye gives a larger plasma volume than subsequent injection is not in accord with generally known facts and indeed Cruickshank and Whitfield failed to provide obvious evidence in support of their claim. Bonnycastle (25) and Campbell, Sokalchuck and Penman (26) have tested the claim by repeated determinations with T-1824 and find no indication of greater dye loss on the first than on subsequent injections. Any worker with considerable experience with the dye method could scarcely avoid proving to his own satisfaction that the values obtained by repeated determinations are consistent. Yet it must be conceded that the evidence from repeated injections, however well the results agree, does not definitely exclude the possibility of an initial rapid loss of dye during the mixing period. If some mechanism rapidly extracted a constant fraction of the injected dye, regardless of the dose, the results would still check. Furthermore, actual changes in plasma volume between injections might conceivably mask the effects of differences in the fraction of dye so lost. The latter objection is eliminated in the present study by simultaneous measurements with T-1824 and antigens of large molecular weight. Since it is extremely improbable that the dye and antigens would all be rapidly lost during the mixing period, and to exactly the same degree, one is led to conclude that the dye-dilution as determined by extrapolation of the disappearance curve must be a valid measure of the plasma volume.

#### SUMMARY AND CONCLUSIONS

By means of simultaneous measurements it is found that the volume of distribution of T-1824 is identical with that of bovine albumin, bovine globulin and of the polysaccharide SIII (correlation coefficient 0.954). From these results it can be stated with considerable certainty that: *a*) initial rapid loss of significant amounts of T-1824 by staining or phagocytosis does not occur, *b*) conventional extrapolation of the disappearance curve on a semilog plot corrects for the amount of dye lost during the mixing period, and *c*) the volume distribution of T-1824 is a true measure of the plasma volume.

*In vitro* tests with T-1824 and antigen show that the true relative cell volume is on the average  $3\frac{1}{2}$  to 4 per cent less than the hematocrit value obtained by centrifugation for 30 minutes at  $1500 \times g$ .

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# EFFECTS OF MASSIVE TRANSFUSION AND HEMORRHAGE ON BLOOD PRESSURE AND FLUID SHIFTS<sup>1</sup>

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THE effect of transfusion in the treatment of shock has been extensively studied, but the effect of transfusion in *normal animals* has been of less concern to experimentalists. It has, therefore, been the principal purpose of the present study to characterize the changes in circulatory function following massive transfusions into normal animals. Various effects occurring in the donor animals, as a result of hemorrhage, however, have also been recorded and are presented briefly in addition to the transfusion effects.

## METHODS

A total of 74 dogs lightly anesthetized with sodium pentobarbital anesthesia were used in these experiments. This included 40 relatively large dogs used as donors and 34 relatively small dogs used for the transfusion experiments.

Transfusion was performed in the majority of the instances by bleeding the donor from a cannulated femoral artery into an oiled chamber (fig. 1) and then forcing the blood into the femoral artery of the recipient. The arterial pressures were measured by a mercury manometer connected directly to the chamber, and readings were taken from each dog immediately before each transfusion, care being taken to allow neither blood loss nor blood gain during or before the measurements. Immediately following each transfusion the system was flushed with one per cent citrate solution followed by air to eliminate the citrate. Using this system increments of blood (up to 100 cc. of blood each time), without the addition of anticoagulants, could be given over a very long period of time. Heparinized blood, without use of the above system, was employed in 8 experiments with essentially the same results, however.

Transfusion reactions, with resultant severe urticaria and fall in blood pressure, occurred in 6 out of 34 transfused animals, and the results were discarded.

Various measurements were made as follows: red blood cell counts with the hemacytometer; hemoglobins, spectrophotometrically; hematocrits, in Wintrobe tubes spun until the level was constant; and plasma proteins, calculated from the plasma density which was in turn determined by either the copper sulfate or falling drop method.

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For purposes of analyzing the results, the control blood volume of each animal in cc. has been considered as one-twelfth the body weight in grams, and the results are presented in terms of the control blood volume of the transfused or hemorrhaged animal, considering this value to be unity in each case.

### RESULTS

*A. Effect of Volume of Blood Transfusion on Change in Arterial Pressure.* Fifteen dogs, averaging 5.6 kg. in weight, were transfused without reaction with sufficient blood to increase the total blood in each animal to an average of 1.97 times its con-

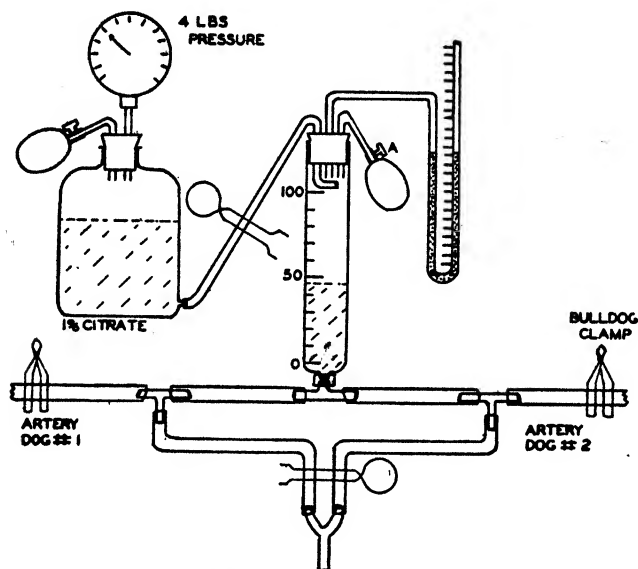


Fig. 1. APPARATUS FOR RAPID TRANSFER of small quantities of whole, coagulable blood from one animal to another.

trol value, the range being from 1.4 to 2.64. The curves in figure 2, designated *A*, *B*, *C*, and *D*, represent the blood pressure changes in representative experiments, and the dashed curve, *E*, is an average curve obtained in the 15 experiments. It will be noted that the average maximum increase in blood pressure was 17 mm. Hg which occurred when the total blood in the animal reached an average of 1.8 times the control value.

*B. Effect of Volume of Blood Hemorrhage on the Change in Arterial Pressure.* Eighteen dogs, averaging 13.5 kg. in weight, were bled at various rates until the arterial pressure fell rapidly, and the pressure was plotted against the fraction of blood volume still remaining in each animal's body. Four representative curves, designated as *F*, *G*, *H* and *I*, are shown in figure 2, and an average of all 18 curves is given by the dashed curve, *J*, in the figure. As noted in the solid curves, the blood pressure usually remained fairly constant when blood was first removed from the dog but

fell rapidly when the amount withdrawn reached a critical point. This phenomenon is not evident from the average curve, *J*, because 5 out of the 18 animals showed precipitous decreases in blood pressure immediately following removal of the first quantity of blood. Blood pressure in the average animal fell to one-half the control value when 0.4 of his blood has been removed.

*C. Effect of Rate of Transfusion and Hemorrhage on Blood Pressure.* The rate of transfusion in the 15 experiments noted in section A varied between 0.005 and 0.1 control blood volumes per minute. The rate of hemorrhage in the experiments of section B varied between 0.003 to 0.1 control blood volumes per minute. At these

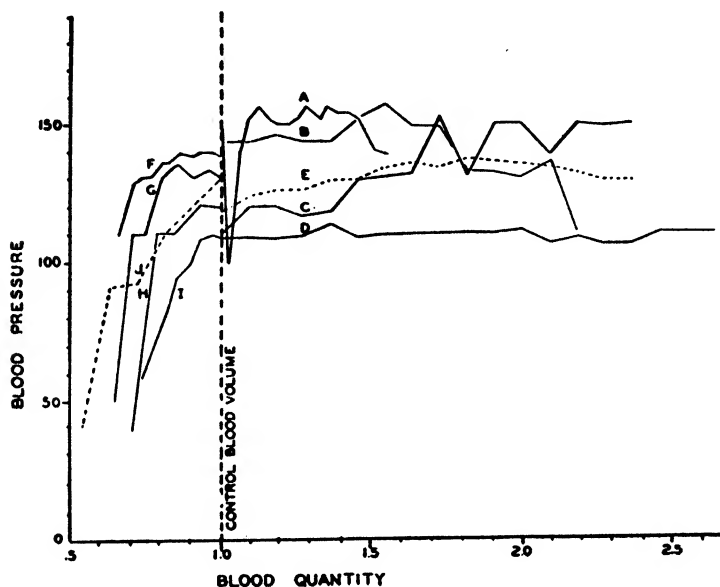


Fig. 2. EFFECT OF TRANSFUSION and hemorrhage on blood pressure in normal animals. Transfusion is represented to the right of the control blood volume line and hemorrhage to the left. Curves *E* and *J* are average curves of many determinations; the others are for individual animals.

relatively slow rates, no effect of rate per se could be discerned from the records, and the changes in blood pressure appeared to be entirely, or almost so, a function of volume of blood injected or removed.

*D. Effect of Denervating the Pressoreceptors on the Response of Blood Pressure to Blood Volume Changes.* In figure 3, curve *A*, is an average curve of 2 transfusions into dogs whose carotid sinuses had been stripped and vagi cut immediately before each experiment. Curve *B* is the average curve of 2 hemorrhage experiments performed on animals similarly prepared immediately prior to each experiment. In 3 additional animals whose carotid sinuses had been stripped for one inch in all directions 2 weeks prior to the experiment and the vagi cut immediately before the experiment, transfusion curves were comparable to those shown in figure 2 from normal animals. In all 5 of the transfused animals, however, severe pulmonary congestion and cardiac

irregularities—particularly a series of skipped beats—occurred. One animal hemorrhaged severely from the lung, though the blood in him at the time was only 1.6 times the control value.

*E. Effect of Spinal Anesthesia on the Response of Blood Pressure to Changes in Blood Volume.* Curves I, II and III of figure 4 are actual results and curve A the average result of 3 transfusions into animals which had been given sufficient one per cent procaine at L-3 to cause complete paralysis of the spinal cord and even paralysis

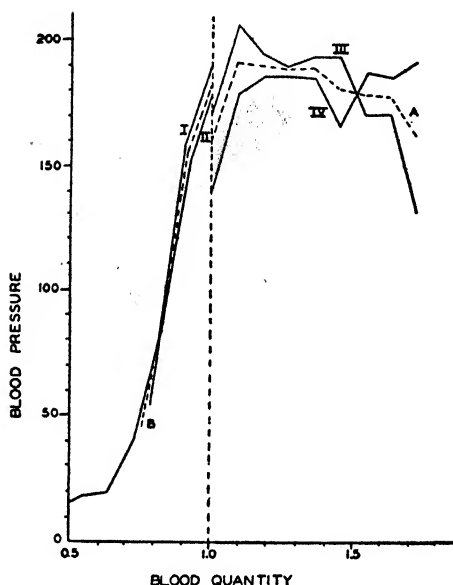


Fig. 3. EFFECT OF TRANSFUSION and hemorrhage on blood pressure in pressoreceptor denervated animals. Transfusion is represented to the right of the control blood quantity of 1.0 and hemorrhage to the left. Curves A and B are average curves; the others represent results from individual experiments.

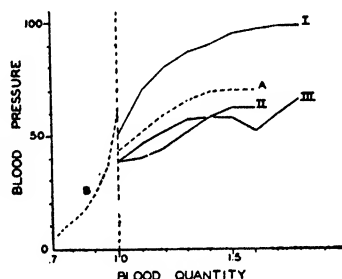
of respiration (artificial respiration was used). Curve B illustrates the average effect of bleeding 5 spinally anesthetized animals. The 5 actual curves were so close together that it was not possible to portray them all in a single graph.

*F. Effect of Splanchnicectomy on the Response of Blood Pressure to Changes in Blood Volume.* Bilateral lumbodorsal sympathectomy with removal of all splanchnics and the chain from D-8 through D-12 was performed in 12 dogs 2 weeks before each experiment, and transfusion and hemorrhage were each performed six times. Comparable amounts of blood at comparable rates as those represented in the normal records of figure 2 were transfused into and bled from the animals. The curves of blood pressure changes during transfusion and hemorrhage were so nearly the same as those obtained in normal animals that no general differences could be discerned.

*G. Effects of Massive Transfusion on Fluid Balance.* In 7 animals receiving transfusions at an average rate of 0.03 blood volumes per minute, the final total quantity of blood in each animal was calculated by adding the animal's blood volume and the transfusion blood volume. An average of these calculations is shown in section a of table 1. By algebraic addition, and assuming no fluid loss from the circulatory system, the theoretical average hematocrits, hemoglobins, red blood cell counts, and plasma proteins at the end of the transfusions were also calculated from the known values for the animals' normal bloods and for the transfusion bloods. These calculated values are shown in section b of table 1. The average values actually determined at the end of transfusion are also shown in section b, and it will be noted that all of the blood constituents became much more concentrated than would have

been true should no fluid have been lost from the circulation. The final blood volume was then calculated from the increase in concentration of each the hematocrit, the hemoglobin, and the red blood cell count. Subtracting these final blood volumes from the total blood volumes, the fluid loss was determined and is given in *section c* of table 1. Expressed in percentage of the blood put into the animals the loss of fluid averaged 63 per cent. Expressed in percentage of the plasma portion of the transfused blood the fluid loss averaged 113 per cent. In *section d* of table 1, the average quantity of plasma protein which was lost following transfusion is calculated. Expressed in percentage of the quantity of plasma protein injected during transfusion, the quantity lost through the capillary walls averaged 91 per cent. (The relatively low hemoglobins and high plasma proteins noted in table 1 have been a constant finding in the type of dog used in these studies, i.e. scavenger dogs brought in from rural areas. Hemoglobins, determined by three different methods in approximately 75 of these dogs, have verified this low value. The high proteins, however, have been determined only by specific gravity techniques as noted under methods and have not

Fig. 4. EFFECT OF TRANSFUSION and hemorrhage on blood pressure in animals with total spinal anesthesia. Transfusion is represented to the right of the control blood quantity of 1.0 and hemorrhage to the left. Curves A and B are average curves; the others represent results from individual experiments.



been determined by protein nitrogen methods. It must be emphasized that it is the change in these values which has been the important factor in the present studies, and it is felt that the calculated results represent essentially a true picture. The use of dye methods in these experiments has been considered too inaccurate for consideration because of the rapid protein loss, and radioactive iron methods, depending on the combination of the iron in hemoglobin, offer few advantages over simple studies of hemoglobin.)

**H. Effect of Massive Hemorrhage on Fluid Shifts.** Changes in the concentration of blood constituents in 6 hemorrhage experiments were studied. An average of 39 per cent of the total blood was removed at an average rate of one per cent per minute. At the end of the hemorrhages the hematocrits of the bled dogs had increased an average of 3 per cent, the red blood cell counts had increased an average of 3 per cent, the hemoglobins averaged no change, and the plasma protein had decreased an average of 6 per cent.

**I. Miscellaneous Effects of Massive Transfusion.** The venous pressure as measured from the external jugular vein during 9 transfusion experiments rose immediately after each small increment of blood was transfused into the animal, but no sustained increase in venous pressure was recorded until an average of 80 per cent as much blood as the animal already had in his body had been transfused into him. Follow-

ing this quantity, the venous pressure rose rapidly to an average of 13 cm. water pressure above normal in the 3 animals which received transfusions equal to 150 per cent of the control blood volume. Venous pressures measured by the more accurate method of intra-auricular catheterization during 7 other transfusion experi-

TABLE I

## a) Average fluid and protein leakage from circulation following transfusion

Blood volume of average transfused animal.....	100%
Total average transfusion (in per cent of normal).....	120%
Total average final quantity of blood in animal.....	220%

## b) Average calculated values (provided there were no capillary leakage) and actual measured values at the end of transfusion

	HEMATOCRIT (%)	Hb (GM. %)	RBC (MILLIONS)	PL. PROT. (GM. %)
Calculated by algebraic addition of transfused blood and animal's control blood values.....	40.4	10.9	4.85	9.3
Actual final values.....	61.0	15.7	8.00	11.1

## c) Average fluid lost from circulation following transfusion

	EXPRESSED IN PERCENTAGE OF WHOLE BLOOD TRANSFUSED	EXPRESSED IN PERCENTAGE OF PLASMA TRANSFUSED
Determined from increase in hematocrit concentration...	62	110
Determined from increase in hemoglobin concentration...	56	100
Determined from increase in red blood cell concentration.....	72	129
Average.....	63	113

## d) Effects of transfusion on plasma proteins

	%
Quantity of protein in normal animal.....	100
Quantity of protein in transfused blood.....	158
Total quantity of protein in animal's blood at end of transfusion if no losses should have occurred.....	258
Actual quantity in animal's blood stream as determined from average quantity of plasma remaining in the blood stream times the plasma protein concentration of 11.1%.....	114
Loss through capillary wall.....	144
Loss of plasma protein expressed in percentage of that transfused into the animals	$144/158 \times 100 = 91\%$

ments, using quantities of heparinized blood up to 50 per cent of the control blood volume, showed a sustained rise of not over one cm. water pressure.

In addition to the pulmonary congestion and cardiac irregularities found in pressoreceptor denervated animals, these same effects were found to a much lesser degree in normal animals which had received transfusions equal to or greater than their control blood volumes. Also, the abdomen and skin showed swelling in every instance, though no excess intraperitoneal fluid was found. In approximately one-third of the animals, bloody diarrhea occurred either during the course of the trans-

fusion or within 12 hours thereafter, and autopsied animals often showed minor infarcts throughout the abdominal viscera with occasional major infarcts as large as an inch in size on the surface of the kidney and liver.

After a large quantity of blood had been transfused into an animal it was never possible to remove more than one-half the quantity of blood injected without throwing the animal into shock.

#### DISCUSSION

The effects on circulation of quantity of blood in the body have been studied in these experiments by beginning at normal blood quantity and bleeding some dogs while transfusing others. Perhaps it would have been better to study animals by beginning at zero blood volume and transfusing them far beyond the control blood level, but this method was found to cause momentary shock and considerable detrimental effect on the ensuing experiment. Yet, from the results obtained with the present methods, and utilizing the findings of other investigators, the effect of increasing blood quantity in the animal, beginning at zero quantity, may be reconstructed as follows (the principles of this schema are well known, the present studies having been performed mainly to add quantitative data).

Until the blood quantity reaches 30 to 40 per cent of the control value, the vessels remain partially collapsed, and the blood pressure remains essentially zero. As the quantity of blood increases above this point, the vessel walls actually begin to distend causing pressure to develop in all chambers of the circulatory system. The pressure in the venous system and the heart becomes sufficient that propulsion of blood begins, and thereafter, the pressure in the arteries is greater than in other portions of the system. The capillary pressure at this point is low, and the colloid osmotic pressure is high so that no fluids leak from the circulatory system, and if anything, fluid is pulled in from the interstitial spaces. Actually, in the present studies, as well as in work by others, it has been found that the inflow of fluid from the tissues during low blood volume is very slow (1) unless the animals have been specifically hydrated prior to hemorrhage (2) or are given water by mouth or otherwise after the hemorrhage (3).

As the quantity of blood increases toward the normal value, the vessels become more distended, the cardiac output becomes greater, and all of the pressures in the circulatory system increase. Until the pressure in the capillaries has increased sufficiently to overcome osmotic pressure gradients, all of the blood injected into the animal remains in the circulatory system. Therefore, up to this point there is an increasing filling of the circulatory system and an increasing pressure, but when the hydrostatic pressure acting across the capillary wall exceeds the osmotic gradient, fluid loss begins to occur, the actual volume of circulating blood does not increase greatly beyond this point despite continued transfusion, and the blood pressure has reached a point of relative stability.

The extreme flatness of the blood pressure plateau as blood quantity increases above the control quantity has been very surprising, for transfusions as great as 1.62 times the animal's original blood volume have been given. This is equivalent to approximately 9 liters given to a human being. Assuming that the methods of



blood volume calculation were adequate in these experiments, it was equally as surprising to find the extreme degree of fluid loss—namely, 63 per cent of the transfused blood or 113 per cent of the plasma portion of the transfused blood—all of this occurring in approximately 40 minutes, the average duration of the experiments. Furthermore, these calculated losses were very consistent from experiment to experiment and have been confirmed in an additional series of experiments concerning very rapid transfusion (4). The calculated loss of 91 per cent of the transfused plasma protein in this same 40 minutes indicates that the fluid leaving the plasma and entering the interstitial spaces carried with it a relatively large concentration of protein.

That large quantities of fluid may leave the circulation very rapidly when the capillary pressure is high has been shown by numerous workers and has recently been extensively reviewed by Landis and Hortenstine (5). Furthermore, others have shown that when the blood volume is increased by infusion of various solutions, large quantities of protein may also be lost very rapidly (6-9). On the other hand, the inflow of fluid from the tissues following hemorrhage has been found in the present studies, as well as in studies by others (1-3), to be very slow. The great difference between the rate of inflow following hemorrhage and the rate of outflow following transfusion is difficult to reconcile with the linear relationship between capillary pressure and the rate of inflow and outflow found by Landis *et al.* (10-13), but obviously, there are many factors which have not yet been elucidated.

On the basis of the schema that has been presented, the level at which blood volume is regulated would be to a great extent a function of the physical capacity of the circulatory system. The body is in reality being constantly transfused with water from the gut, plasma proteins from the liver, and very slowly, cells from the bone marrow. It is reasonable to expect the blood volume to increase to that quantity which will cause just sufficient pressure in the capillaries to bring about hydrostatic and osmotic equilibrium with the interstitial fluids. Excess fluids thereafter will rapidly be lost from the capillaries. The point of equilibrium in the quantity of red blood cells, however, is dependent on other factors. It has been shown that plethoria in rabbits, as a result of daily injection of blood, results in the maintenance of a constant total plasma volume but an increase in red blood cell volume (14). Also, in patients with polycythemia vera, the total plasma volume remains relatively normal, though the great increase in red blood cells causes a large total blood volume (15). Furthermore, in the present transfusion experiments, the plasma volume at the end of transfusion was nearly normal (slightly less than normal), but the total blood volume averaged approximately 35 per cent greater than normal because of the large quantity of red blood cells. Why the excess presence of red blood cells does not actually cause the plasma volume to decrease proportionately is a problem yet to be solved.

Finally, and very important, the level of the blood pressure plateau during slow transfusion appears to be greatly dependent on nervous activity. In 2 animals with denervated pressoreceptors, and therefore, presumably having increased sympathetic activity, the plateau averaged 54 mm. Hg above the level of the normal plateau.

In 3 spinally anesthetized animals, in which the sympathetics were presumably inactive, the plateau averaged 70 mm. Hg below the normal. These results in pressoreceptor denervated and spinally anesthetized animals have been confirmed in a slightly different manner by rapid transfusions four times into denervated animals and 5 times into spinally anesthetized animals (4). The major presumption in the schema which has been discussed is that the capillary pressure seeks a relatively constant level. It is logical, therefore, that the degree of sympathetic activity, and resultant arteriolar contraction, should determine how much greater will be the arterial blood pressure than the capillary pressure. Thus, the arterial blood pressure in reality is determined by two factors: first, the level of the blood pressure plateau resulting from intrinsic circulatory adjustment, and second, the vasoconstrictor and cardiac excitator factors, either nervous or humoral, which may elevate the pressure still higher. The importance of the first of these factors is often neglected.

#### SUMMARY

Transfusion and hemorrhage at rates less than 0.1 control blood volumes per minute have been studied in 74 dogs. At these slow rates the blood pressure appeared to be a function mainly of blood quantity in the animal at any given time and relatively little affected by the *rate per se* of transfusion or hemorrhage. Increasing the quantity of blood in a dog to as much as 2.62 times the control value caused very little increase in blood pressure, the maximum increase in pressure being an average of 17 mm. Hg when the average quantity of blood in the animals reached 1.8 times the control value. At greater quantities than this the pressure fell. Denervating the pressoreceptors, presumably followed by excess sympathetic activity, resulted in a blood pressure plateau averaging 54 mm. Hg above normal when these animals were given slow but massive transfusions. Complete spinal anesthesia, presumably followed by dormant sympathetics, resulted in a blood pressure plateau averaging 70 mm. Hg below normal when the animals were given slow but massive transfusions.

Hemorrhage failed to cause either marked or rapid shifts of fluid between the interstitial spaces and the blood stream. From determinations of hematocrits, hemoglobins, and red cell counts, it appeared that during massive transfusion occurring over an average period of approximately 40 minutes an average of 63 per cent of the transfused whole blood and 113 per cent of the plasma portion of the transfused blood was lost from the circulation by the time transfusion was complete. Relationship of the above findings to the basic physiology of blood volume and blood pressure control in the normal animal is discussed.

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# EFFECTS OF CONTROL OF CARDIAC WORK UPON CORONARY FLOW AND $O_2$ CONSUMPTION AFTER SYMPATHETIC NERVE STIMULATION<sup>1</sup>

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PREVIOUSLY (1) the mechanism of coronary flow increases following stimulation of the cardiac accelerator nerves was studied. Because of the behavior of the coronary flow in fully dilated vessels the conclusion was reached that a maximum of 30 per cent increases in flow can be accounted for by decreases in the systole/cycle ratio. However no significant increases in flow occur as a result of decreases in cardiac volume or intraventricular diastolic pressure. The so called massaging action due to the increased vigor of contraction is not a factor nor is the possible decrease in intramyocardial pressure. In confirmation of the work of Gregg and Shipley (2, 3) it is felt that a true coronary dilation is necessary to explain the major increase in flow.

The mechanism of this coronary dilation is still obscure. It is due 1) to the direct effect of the sympathetic nerves upon the coronary arteries or 2) to the increased myocardial production of vasodilator metabolites or 3) perhaps a combination of 1 and 2. Gregg and Shipley (2, 3) showed that sympathetic nerve stimulation produced an increase in cardiac output, cardiac work, cardiac  $O_2$  consumption and vigor of contractions. The problem, therefore, resolves itself into a separation of direct cardiac from possible direct coronary effects of the accelerator nerves. Thus far no crucial experiments have been done either with drugs or by mechanical means.

It occurred to us that if myocardial  $O_2$  consumption could be kept constant during nerve stimulation the behavior of the coronary arteries could be studied independently. Since  $O_2$  consumption depends upon cardiac work, the control of cardiac work by the reduction of cardiac output seemed likely to produce the desired conditions. This paper is a report of the results of such a study.

## METHODS

Dogs were anesthetized with morphine and sodium pentobarbital. Under artificial respiration the left chest was opened between the third and fourth ribs. The blood was rendered noncoagulable with heparin. Figure 1 shows the apparatus for coronary flow and cardiac output measurements. Blood was led from the left common

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carotid artery through two rubber flap valves. A pump acting between the valves forced the blood into a constant pressure chamber. A rubber membrane separated the fluid in the pump from the blood. Constant perfusion pressure was used so as to be able to evaluate critically vasomotor changes. Perfusion pressure was maintained constant by means of air flowing through the chamber above the blood. The air escaped through a hole in a rubber membrane stretched over the chamber. Adjustment of the air inflow produced any perfusion pressure desired. The perfusion pressure was read directly on a mercury manometer and recorded with a Gregg optical manometer (4). The blood was led from the chamber through a Shipley recording rotometer (5) and then into the common left coronary cannulated with the special cannula (6) introduced through the left subclavian artery. The cannula was firmly tied into the left common coronary artery.

The cardiac output was measured with a large Shipley recording rotometer placed in the thoracic aorta with a special cannula (fig. 1*L*). A large air chamber was

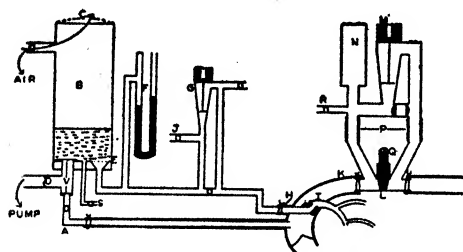


Fig. 1. CORONARY PERFUSION and cardiac output apparatus. *A*, Cannula through left carotid into aorta; *B*, constant pressure chamber; *C*, rubber membrane for escape of air; *D*, rubber membrane separating pump from blood; *E*, outlet from pressure chamber; *F*, mercury manometer to indicate perfusion pressure; *G*, Shipley recording rotometer; *H*, cannula through left subclavian artery; *I*, cannula tied in ostium of common left coronary artery; *J*, outlet for optical recording of

perfusion pressure; *K*, aorta; *L*, special cannula tied in thoracic aorta; *M*, large Shipley recording rotometer; *N*, air chamber; *P*, rubber connections; *Q*, screw for opening or closing aortic cannula; *R*, outlet for optical recording of aortic pressure; *S*, tube for arterial blood samples; *V*, rubber flap valves.

used to minimize the excursion of the float. Aortic pressure was recorded from this rotometer with a Gregg manometer (4). In some cases the right subclavian was ligated about the left carotid cannula. This gave then all the cardiac output except for the right and left coronary flow. In the results the left coronary flow was added to the metered output value. Both rotometers were calibrated with the blood of the animal.

The coronary sinus was cannulated through the right auricle and the blood returned to the left jugular vein. The cannula was fixed in place by an inflated balloon at the cannula tip. The left cardiac accelerator nerve was isolated and usually sectioned from the stellate ganglion and stimulated with an electrodyne stimulator at a rate of 50 per second. Cardiac output was controlled by the simultaneous inflation of balloons in the inferior and superior cavae introduced through the right jugular vein. Blood samples were drawn simultaneously from the perfusion chamber and coronary sinus cannula and analyzed for  $O_2$  content by the methods of Scholander or Van Slyke. With these techniques the following were measured: Aortic pressure, cardiac output, cardiac work, coronary flow and myocardial  $O_2$  consumption. The kinetic cardiac work factor was found to be too small to be important.

## PROCEDURE

Each experiment consisted of 4 parts: 1) control data were obtained; 2) measurements were made following sympathetic stimulation; 3) a new set of control observations was made, and 4) data were obtained during simultaneous sympathetic stimulation and the control of cardiac output by the inflation of the balloons in the venae cavae.

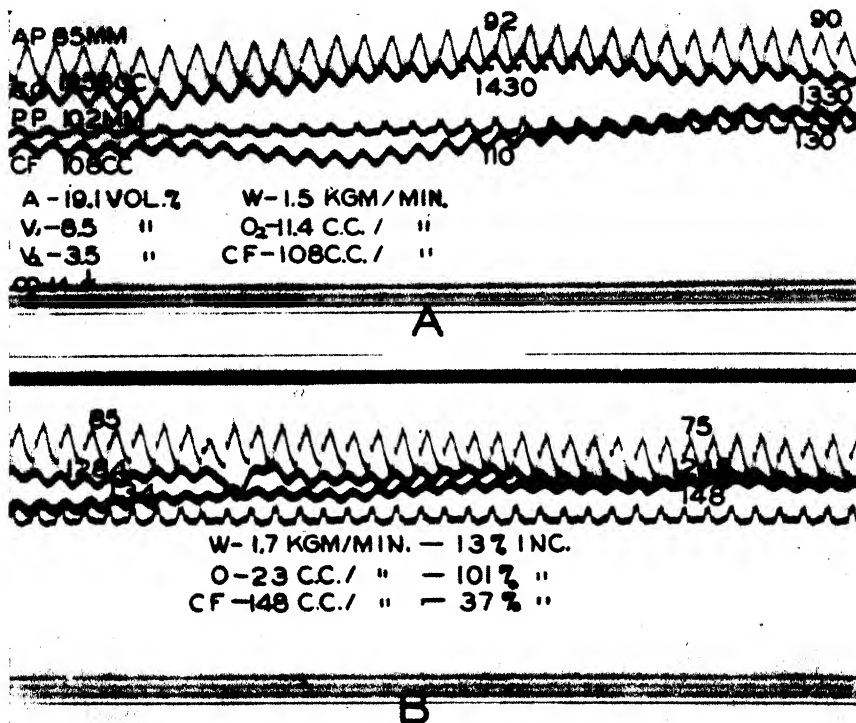


Fig. 2. RECORDS SHOWING TYPICAL RESPONSE TO accelerator nerve stimulation. *A.P.*, Aortic pressure; *C.O.*, cardiac output; *P.P.*, perfusion pressure; *C.F.*, coronary flow. Arrow shows beginning of stimulation. Record *B* is continuation of *A*.

## RESULTS

Figure 2 shows an experiment with measurements before and after nerve stimulation with uncontrolled output. The control values are given in the upper record *A*. The work was 1.5 kg minute, the O<sub>2</sub> consumption was 11.4 cc minute, and the coronary flow was 108 cc minute. The arrow indicates the beginning of stimulation and the lower record (*B*) shows the effects. The work increased 13 per cent. The increase in O<sub>2</sub> consumption was largely met by the decreases in coronary sinus O<sub>2</sub> content from 8.5 to 3.5 vol. per cent. The fact that the O<sub>2</sub> consumption increased out of proportion to the increase in work suggests that sympathetic nerve stimulation alters cardiac metabolism in some way which is not related to external work.

Figure 3 from a different dog shows the events following simultaneous nerve stimulation and reduction of cardiac output. Again the top record (A) shows the control values. Cardiac work was 1.3 kg/minute,  $O_2$  consumption was 8.4 cc/minute and coronary flow was 125 cc/minute. The arrow shows the beginning of nerve stimulation and the inflation of the balloons in the venae cavae. The effects of these procedures are shown in B and C. The lower record (D) shows that in spite of a decrease in cardiac work there were sizeable increases in  $O_2$  consumption and coronary flow of 34 per cent and 24 per cent respectively. The last 3 lower records (E, F and G) show

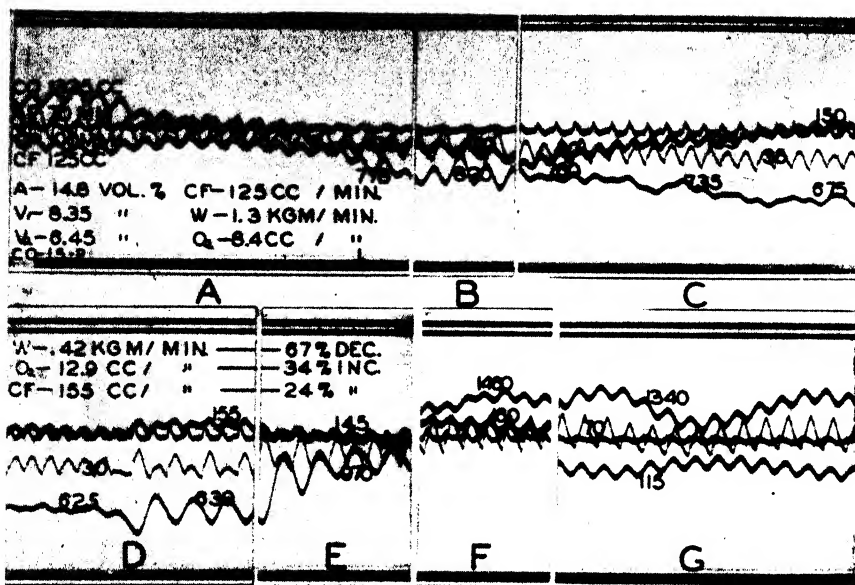


Fig. 3. RECORDS SHOWING TYPICAL RESPONSE to accelerator nerve stimulation and simultaneous decrease in cardiac output. Curves are labeled as in figure 2. Arrow in A shows beginning of nerve stimulation and inflation of balloons in venae cavae; B and C, continuation of A showing increasing coronary flow and decreasing output; D, taken during blood sampling; E, F and G, changes after deflation of balloons in venae cavae.

that the removal of the nerve stimulus and the return of cardiac output to normal reduce the coronary flow to 115 cc/minute, even slightly below normal. These data lend strong support to the view that the increased  $O_2$  consumption following nerve stimulation is not due to increases in cardiac work.

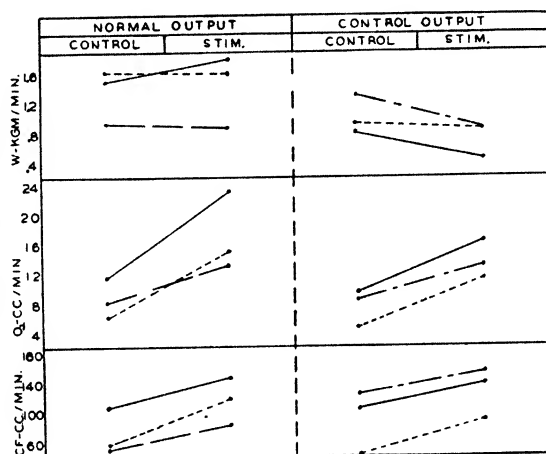
Figure 4 is a composite diagram from 3 experiments showing the changes in cardiac work,  $O_2$  consumption and coronary flow due to nerve stimulation with normal and controlled cardiac output. The left upper graph indicates that nerve stimulation elevated cardiac work in one instance and then not markedly. The left middle graphs show increases in  $O_2$  consumption in all 3 experiments. The left lower graphs show similar increases in coronary flow. The data on the right show the effects in the same 3 dogs of simultaneous nerve stimulation and reduction in cardiac output. In

each case it may be seen that in spite of marked reduction in cardiac work both  $O_2$  consumption and coronary flow were increased. The increases are not necessarily so marked as the control experiments but they are consistent and significant.

#### DISCUSSION

It is obvious that these experiments do not serve to separate direct nerve effects from metabolic effects upon the coronary arteries. Sympathetic nerve stimulation must in some way augment the metabolic demands of the myocardium, thereby producing inefficiency. Gollwitzer-Meier (7) showed after adrenaline there was an increase in  $O_2$  consumption even after cardiac work had returned to normal—thereby coining the phrase “adrenaline is an anoxating agent.” Certainly sympathetic nerve stimulation produces an adrenaline-like substance and an adrenaline-like myocardial effect.

Fig. 4. SUMMARY OF 3 experiments. On *left* is shown changes in cardiac work in kg/min.; cardiac  $O_2$  consumption in cc/min.; and coronary flow in cc/min. following nerve stimulation with uncontrolled output. On *right* same data are given following nerve stimulation and control of cardiac output. Each set of similar lines represents same experiment.



The exact mechanism of this anoxating effect is not clear. It may be due to an intrinsic change in myocardial type and/or rate of metabolism or, to a true mechanical inefficiency. The small heart seen after nerve stimulation (1) theoretically should be a more efficient organ. However, there is a possibility that because of its decrease in size during mid-systole the endocardial surfaces meet and muscular contraction continues much as a tightly clenched fist. Such a process would produce no measurable work but certainly would require oxygen. Some evidence for this view is given in the phasic flow records of Gregg and Shipley (2) which show an increase in systolic back flow in the presence of a normal or lower aortic pressure. More recently the studies of Gauer (8) suggest a similar phenomenon in shock.

The conclusion then is reached that cardiac accelerator nerve stimulation produces an adrenaline-like effect in the myocardium which increases the  $O_2$  consumption and coronary blood flow in spite of marked experimental reduction of cardiac work. Finally, it may be stated that it is not justifiable to attribute changes in coronary flow to the direct action of nerves or drugs upon the coronary arteries unless the myocardial metabolic requirements are controlled.



## SUMMARY

Left coronary blood flow, cardiac work and myocardial  $O_2$  consumption were measured in open-chest dogs before and after accelerator nerve stimulation with normal and controlled cardiac output. Nerve stimulation markedly increases myocardial  $O_2$  consumption and coronary flow and produces small increases in work. Even after cardiac work is markedly reduced nerve stimulation elevates cardiac  $O_2$  consumption and coronary blood flow. Sympathetic nerve stimulation by releasing an adrenaline-like substance renders the heart anoxic and inefficient. The interpretation of coronary vasomotor changes requires knowledge of the myocardial metabolic requirements.

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# EFFECTS OF HEMOLYZED BLOOD ON CORONARY BLOOD FLOW<sup>1</sup>

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IN RECENT years there have been many reports concerning vasoconstrictor substances in blood; however, few have described vasodilator substances present in or released from the constituents of whole blood under various circumstances. The vasoconstrictor substance of clotted blood called 'thrombocytin' by Reid (1) and 'SMC' by Zucker (2) has been carefully studied. Serotonin has been isolated and crystalized by Rapport *et al.* (3). Freund isolated a vasodilator substance from defibrinated blood which he called 'Frühgift' (4) and Frey (5) has found Kallikrein in blood. In the course of other experiments, we accidentally found that a potent vasodilator substance was released from whole blood when it was rapidly injected into a cannulated coronary artery. The mode of production and identification of this substance form the basis of this report.

## METHODS

In all the following observations open chest dogs, under artificial respiration, weighing 14 to 24 kg. were used. They were anesthetized with sodium pentobarbital intravenously and the blood rendered noncoagulable with heparin and/or chlorozol or pontamine fast pink. The left common coronary artery was cannulated with a special cannula (fig. 1) through the cephalobrachial artery and the aorta. The mean total left coronary blood flow was measured with a Shipley recording rotameter (6) and photographic records made as described in detail elsewhere (7). In most experiments arterial pressure, perfusion pressure, and total left coronary blood flow were recorded; in those in which the perfusion pressure was not recorded, the left common coronary was perfused at arterial pressure. Most of the blood samples used as test substances were heparinized, though some were withdrawn from the dog after heparin and/or chlorozol or pontamine fast pink had been administered. All injections were made into the rubber connection between the rotameter and the coronary artery cannula unless otherwise specified.

## RESULTS

While studying various factors influencing coronary blood flow in dogs, it was observed that small amounts of blood rapidly injected through a small bore needle

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into the output side of a Shipley recording rotameter produced transient but quite marked increases in blood flow. These increases were from 50 per cent to slightly over 100 per cent of the control flows. The onset of the effect was about 2.5 seconds after the injection depending on the rate of flow and the distance from the site of injection to the coronary vascular bed. The duration of effect was from 5 to 30 seconds and was occasionally followed by a slight but brief decrease in flow.

Figure 2 illustrates such an effect. Whole blood, 0.2 cc., was rapidly injected into the rubber connection to the coronary artery cannula. There was an increase in blood flow from 58 cc/minute to 129 cc/minute. The maximum effect was seen in 11 seconds and there was no significant change in arterial or perfusion pressures. In attempting to explain such changes, the effect of the increased pressure within the system must be considered. It is highly improbable that the rapid injection of such a small volume could forcibly distend the coronary vascular bed and increase the blood flow for such a period of time. It was found that when the perfusion pressure was suddenly raised for a period of one second, there was an immediate increase in blood flow; and as soon as the pressure was reduced to normal, the flow returned to

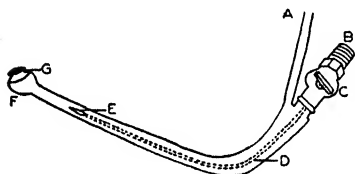


Fig. 1. CANNULA USED to cannulate the left common coronary artery through the cephalo-brachial artery and aorta. A: arterial blood inflow tube; B: connection to optical manometer to record aortic pressure; C: stopcock; D: large bore needle soldered to side of brass cannula; E: opening of needle which remains in aortic lumen; F: flange which prevents insertion of cannula far enough to occlude one of the branches of the left common coronary artery; G: groove to tie cannula in place with a ligature around the left common coronary artery.

normal. This shows that the increase in flow due to forcible distension of the vessels ceases as soon as the pressure is returned to normal.

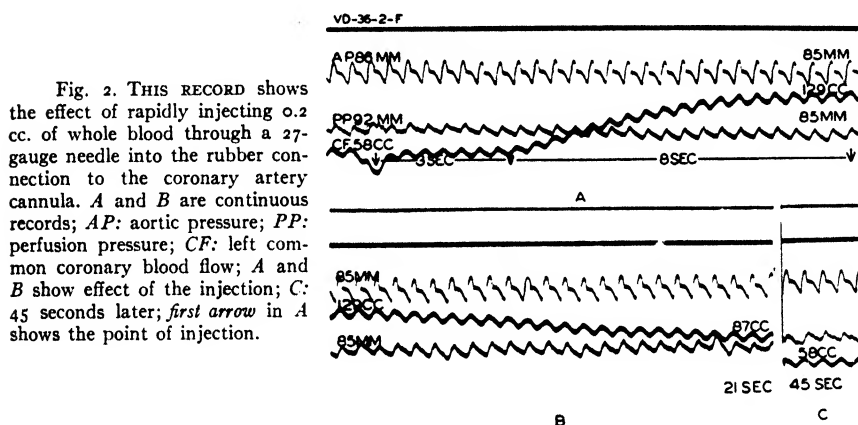
In order to determine the role of the injected substance, the same experiment was repeated by rapidly injecting 0.2 cc. of normal saline through a 27-gauge needle into the system (table 1). The blood flow was increased from 37 to 68 cc/minute without significant change in arterial or perfusion pressure. It was thought that this might be due to viscosity changes of the blood, but when approximately the same amount of whole blood, arterial serum, or coronary sinus serum was rapidly injected into the system, significant increases in blood flow were observed (table 1).

It was then observed that when approximately 0.2 cc. of fresh whole blood, red blood cells, blood serum, or saline were slowly injected into the system, no significant change in blood flow occurred. When similar amounts of these substances were rapidly injected into the system, an increase in flow invariably occurred (table 1). The one factor common to all these injections was the forceful ejection of the contents of the syringe through a small bore needle into the coronary blood stream. It was felt that this forceful ejection of blood through a small needle might traumatize the red blood cells and that the increase in blood flow resulted from the effect of such cells.

Further studies showed this to be true. A small amount of blood was rapidly

injected through a 27-gauge needle into 10 cc. of blood in a beaker. When this blood was centrifuged gross hemolysis was easily demonstrable, showing that the red blood cells had indeed been mechanically traumatized. When 0.25 cc. of a blood sample prepared in this manner was slowly injected into the system, there was an increase in blood flow from 108 to 217 cc/minute without significant change in arterial or perfusion pressures (table 1). Samples of traumatized blood serum slowly injected into the system caused little or no effect on the blood flow. This demonstrated that red blood cells were mechanically traumatized when whole blood was forcefully injected through a small bore needle, and that such a preparation contained a vasodilator substance; but this did not explain the effects of rapidly injected saline or serum.

*In vitro* experiments were then set up to simulate the injection of whole blood and saline into the coronary blood stream. Whole blood, 40 cc., was withdrawn from



a dog and equally divided into 5 tubes. *Tube 1* served as a control. Then 0.1 cc. of blood was rapidly injected through a 27-gauge needle into *tube 2*. In *tube 3* 0.2 cc. of blood was so treated, *tube 4* 0.3 cc. and *tube 5* 0.4 cc. Each of these samples was tested in the dog by the slow injection of 0.2 cc. of each sample tested. The tubes were then centrifuged and the remaining serum diluted 1:4 with saline. The amount of gross hemolysis in *tubes 2* through *5* was quite striking. This was quantitated by reading the percentage of light transmission at a wave length of  $541 \mu$  in a Coleman Junior Spectrophotometer against the control set at 100 per cent. The results in 3 experiments are shown in figure 3. This shows that the degree of hemolysis produced by this form of mechanical trauma increased with the volume of blood injected into the tube, and that the increase in blood flow was related to the amount of hemolysis. The uncontrolled factor in the rate of injection makes it impossible to obtain absolute ratios from this study; however it does show that the amount of increased blood flow is to some extent dependent on the degree of hemolysis. The striking susceptibility of red blood cells to mechanical trauma was further emphasized by the observation that hemolysis easily seen grossly and measureable in the colorimeter could be

produced by repeating the above procedure substituting saline for blood as the injected substance. The common factor of all injections thus appeared to be the hemolysis of red blood cells.

Further test samples were prepared by hemolyzing small amounts of blood with saponin and distilled water. Both of these samples caused significant increases in

TABLE 1. SUBSTANCES TESTED AND THE CHANGES IN CORONARY BLOOD FLOW PRODUCED

MATERIAL INJ.	RATE OF INJ. <sup>1</sup>	NEEDLE SIZE	VOL. INJ.	MEAN CORONARY BLOOD FLOW			MEAN PERF. PRESS		MEAN AORTIC PRESS		EXPER.
				Control	Max.	Change	Control	Max. fl.	Control	Max. fl.	
		gauge	cc.	cc/min.	cc/min.	cc/min.	mm. Hg	mm. Hg	mm. Hg	mm. Hg	no.
Whole blood	R	27	0.2	58	129	71	92	85	86	85	VD-36-II-F
Whole blood	S	20	0.2	42	43	1	88	88	90	87	VD-36-I-A
Saline	R	27	0.2	60	100	40	89	87	84	85	VD-36-I-G
Saline	S	20	0.2	126	132	6	139	137	139	138	M-19-I-A
Art. serum	R	27	0.2	80	138	58	98	95	97	97	M-19-III-C
Art. serum	S	20	0.25	74	68	-6	96	96	65	68	VD-26-II-A
C.S. serum	R	27	0.2	88	137	49	101	94	99	95	M-19-III-D
C.S. serum	S	20	0.25	70	70	0	97	96	66	66	VD-26-III-A
Art. cells	R	27	0.2	90	147	57	84	77	91	90	M-19-III-G
Art. cells	S	20	0.25	71	71	0	95	96	67	67	VD-26-IV-A
C.S. cells	R	27	0.2	93	163	70	82	77	90	88	M-19-III-H
C.S. cells	S	20	0.25	70	72	2	98	96	68	67	VD-26-V-A
Mech. traumatized blood	S	20	0.25	108	217	109	143	144	115	110	VD-24-III-A
Blood hemolyzed with water	S	20	0.25	80	155	75	105	101	65	65	VD-26-C-2
Blood hemolyzed with saponin	S	20	0.25	70	160	90	105	102	64	62	VD-26-C-1
Protein-free H <sub>2</sub> O ext. beef blood	S	20	0.1	85	165	80	113	110	70	76	M-2-I-B
ATP (0.002 M sol.)	S	20	0.01	60	165	105	108	106	90	94	VD-31-IV-A
Blood ext. for ATP (0.002 M sol.)	S	20	0.01	62	155	93	109	106	87	87	VD-31-IV-B
0.1 cc. blood + 0.5 cc. H <sub>2</sub> O + 9.5 cc. saline	S	20	0.5	138	170	32	138	135	137	139	M-19-I-G
0.5 cc. H <sub>2</sub> O + 9.5 cc. saline	S	20	0.1	60	64	4	90	89	84	82	VD-36-III-D

<sup>1</sup> R: rapidly; S: slowly.

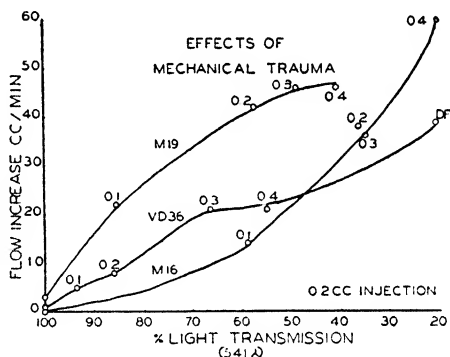
flow without changes in pressure (table 1). They remained potent after the cell fragments were removed by centrifugation.

Although these studies seemed fairly conclusive, the question was raised as to whether the injection of such a small volume of fluid into a moving blood stream could produce enough hemolysis to cause such increases in blood flow as had been observed. One of the experiments in which blood was hemolyzed with water seemed to answer this question. A very dilute solution of blood was prepared in the following manner. Fresh blood, 0.1 cc., was hemolyzed in 0.5 cc. of distilled water. This was then diluted up to 10.0 cc. with normal saline making a relatively isotonic solution.

When 0.05 cc. of this solution was slowly injected into the system the blood flow increased from 138 to 170 cc/minute. The slow injection of the control solution of 0.5 cc. of distilled water plus 9.5 cc. of saline in like amounts caused no significant change in blood flow. The amount of blood used in this test was approximately 1/2000 cc. The activity of such small amounts of blood indicates that the vasodilator substance released from hemolyzed blood is quite potent and active in very small amounts. From these and other experiments it seems quite likely that the injection of saline into the system could produce enough hemolysis to cause the observed effects.

The release of a potent vasodilator substance from the hemolysis of red blood cells was adequately demonstrated in the above experiments. Further studies revealed something of the nature of this substance. Samples of hemolyzed blood allowed to stand in an open container at room temperature lose their effectiveness in about one hour. In one instance, as shown in table 2, the effectiveness was reduced 88 per

Fig. 3. THIS GRAPH shows the relationship of vasoactivity (ordinate) to the degree of hemolysis (abscissa) due to increasing degrees of mechanical trauma. Each curve represents a separate experiment (M 19, VD 36, M 16) on different dogs; the number at each point is amount of blood rapidly injected into 8 cc. of whole blood; point DF (experiment VD 26) represents a sample of freshly defibrinated blood; each sample was tested by the injection of 0.2 cc. (see text).



cent in 33 minutes. This rapid disintegration of the substance suggested enzymatic destruction and militated against the action of inorganic ions. The possibility of the vasodilator substance being acetylcholine or histamine was considered. Against the former was the observation that hemolyzed red blood cells caused an increase in blood flow in dogs given 1.0 mg. of atropine/kg. of body weight. When 0.1 mg/cc. of histamine was added to a preparation of hemolyzed blood and allowed to stand for one hour, there was only a slight decrease in vasodilator activity; whereas the controls without histamine showed an absence or only a slight activity at the end of one hour. When a deproteinated water extract of beef red blood cells (the preparation of this extract will be described later) was boiled for 30 minutes in an acid solution, it was rendered ineffective. Histamine treated in the same manner retained most of its potency. These observations directed our attention to the nucleic acid derivatives and the work of Fleisch.

Although the presence of a vasodilator substance in hemolyzed red blood cells was unknown to us, a review of the literature revealed that the European investigators had observed and studied this phenomenon previously. In 1921 Freund (4) reported a potent vasodilator substance obtained from defibrinated blood which he

called 'Frühgift'. Zipf (8) later obtained a similar substance from blood which he identified as adenylic acid. Later, Fleisch (9-11) in an excellent series of studies, identified adenosine triphosphate (ATP) and probably other adenosine phosphates as being the vasodilator substance in hemolyzed red blood cells.

Following the work of Fleisch we attempted to isolate this vasodilator substance. Four liters of fresh beef blood were centrifuged in the cold and the red cells washed four times in saline. The packed red cells were hemolyzed with a fourfold volume of distilled water. To this solution was added 3 cc. of 0.01 per cent HCl and 29 cc. of 2 per cent NaCl. The solution was boiled and the precipitated protein was removed by filtration. This deproteinated extract of beef blood was found to be quite potent as a vasodilator. An injection of 0.1 cc. of this solution into the system increased the blood flow from 85 to 165 cc/minute with no change in arterial or perfusion pressure. As Fleisch had found, prolonged boiling of this extract in a neutral solution did not destroy it. The dilator in this water extract appeared to be stable whether stored at

TABLE 2. DECREASING ACTIVITY OF HEMOLYZED BLOOD ALLOWED TO STAND IN AN OPEN BEAKER AT ROOM TEMPERATURE<sup>1</sup>

TIME	CORONARY BL. FLOW		INCREASE	PERFUSION PRESS.		ARTERIAL PRESS.	
	Control	Max.	In flow	Control	At max. flow	Control	At max. flow
min.	cc/min.	cc/min.	cc/min.	mm. Hg	mm. Hg	mm. Hg	mm. Hg
3	108	217	109	143	144	115	110
11	110	170	60	124	124	123	112
20	110	161	51	127	125	109	108
33	103	115	13	123	123	102	96

<sup>1</sup> Blood was hemolyzed by rapid injection of 1.0 cc. of blood repeatedly through a 27-gauge needle into 10.0 cc. of whole blood. Approximately 0.25 cc. was slowly injected into the dog preparation in each test.

room or ice box temperature, as opposed to the simple hemolyzed blood preparation which lose their potency quite rapidly. Apparently the method of preparation of this extract destroyed the enzymes responsible for the destruction of the vasodilator substance.

Our observations and those of Fleisch suggested that ATP and/or similar compounds were released when red blood cells were hemolyzed and probably in sufficient concentrations to alter the blood flow when injected directly into the coronary artery. To substantiate this, 4 l. of fresh beef blood were extracted for the barium salt of ATP by the method described by Le Page (12). Approximately 95 mg. of the white, powdered barium salt were obtained. When tested in the dog preparation, 0.01 cc. of a 0.002 molar solution of the hydrolyzed barium salt increased the blood flow from 62 to 155 cc/minute. This compared favorably with commercially prepared ATP (Armour Laboratories) though the effect of our barium salt was slightly more delayed and more transient (table 1). An analysis of this substance on the Beckman Spectrophotometer showed an absorption curve for adenosine compounds. Since our extraction method was for ATP, it seems quite likely that this absorption curve was

primarily due to ATP and possibly some adenosine diphosphate (ADP). The possibility of the presence of adenylic acid is slight since it is ordinarily eliminated in the course of the above preparation. Its absence was further substantiated by a spectrophotometric assay using purified adenylic acid deaminase.

#### DISCUSSION

The vasodilator substance in hemolyzed red blood cells is demonstrated by this study and the previous work of others (9-11, 15). The absolute identification of one or several vasodilators in such a preparation is extremely difficult because of the complex chemical nature of hemolyzed blood. Fleisch (13) from his own very careful chemical and pharmacological studies, concluded that the main vasodilator substance was ATP and/or other closely related compounds. Our observations and the quantitative studies of Fleisch suggest that pure ATP alone is not the sole vasodilator present. Though the chemical identification of a single vasodilator substance is difficult, certain known vasodilators can be readily excluded. Our studies have presented some evidence against histamine and acetylcholine, and Fleisch (13) has presented conclusive evidence against histamine, acetylcholine, and the *P* substance of von Euler and Gaddum (14). From his quantitative chemical studies and pharmacological studies, Fleisch has concluded that the vasodilator substance was not adenosine or adenylic acid.

It seems quite likely that Freund's 'Frühgift' is identical with our vasodilator substance. We have found that freshly defibrinated blood not only is a potent vasodilator, but also is grossly hemolyzed (fig. 3). Defibrinated blood loses its effectiveness with the passage of time in the same manner as our preparations of simple hemolysis. It seems unlikely that defibrinated blood contains two vasodilator substances, one from hemolysis and another from defibrination.

We are unable to fit this vasodilator substance into any physiological role. Fleisch (13) suggested that this substance might be liberated from or 'squeezed out' of red blood cells when trapped in the smallest capillaries, but his experiments did not substantiate this theory. With the same thought in mind we have been unable to demonstrate hemolysis or a vasodilator substance in coronary sinus blood after rapid heart action produced by adrenaline or stimulation of the cardiac accelerator nerves. Before the absence of a physiological role can be assured, more sensitive test methods must be devised.

Although the presence of a vasodilator substance in hemolyzed blood has been extensively reported in the foreign literature, to our knowledge it has largely been ignored in the American. Robertson and Shaw (15) have reported on the adenylic compounds in red blood cells. Phemister and Handy (16) observed the vasodilator effect of hemolyzed blood on the blood flow in the femoral artery of a dog, but did not identify the substance. Though the role of this vasodilator substance in the hemodynamics of the circulation is not apparent, we feel that the investigator should be aware of its presence. The susceptibility of the red blood cells to mechanical trauma has been studied by Shen, Castle and Fleming (17) and re-emphasized in our own observation. In our dog preparations in which blood is circulated through



a 7-cm. plastic rotameter, 36 cm. of rubber tubing, and 2 brass cannulae 10 and 12 cm. long, hemolysis is demonstrable at the end of 2 to 3 hours. In these longer experiments, hemolysis might not take place at a sufficiently rapid rate to cause significant changes in blood flow; but in more acute experiments using other mechanical apparatus, there may be enough hemolysis to cause significant changes in blood flow. The possibility of such hemolysis must be carefully considered in the evaluation of any perfusion data in which blood is circulated through long tubing, squeeze type pumps, differential flow meters and other types of mechanical apparatus. The recent interest in extra corporal circulation using mechanical hearts and oxygenators makes this consideration even more important. Page *et al.* (18) have recently demonstrated a non-adrenaline-like substance in the cross-circulated blood of dogs, and has warned that the variation of results in perfusion experiments may be great and unpredictable. Our data lend weight to his statement.

A recent publication by Deyrup and Walcott (19) has suggested that the vaso-depressor substance released from red blood cells by strongly hypertonic solutions is similar to or identical with the adenosine-like compounds reported here and by Fleisch. The effects of hypertonic solutions on the coronary circulation have been studied in this laboratory, and the incomplete results at the present time tend to support Deyrup and Walcott's conclusions. Experiments are now in progress to determine if such a depressor substance can be 'extracted' from red blood cells by hypertonic solutions without producing hemolysis.

#### SUMMARY

A potent vasodilator substance is released from red blood cells by mechanical trauma when rapid injections of isotonic solutions are made into blood. A substance with identical properties is released from laked blood. Evidence is presented suggesting that this vasodilator substance is most likely adenosine triphosphate and/or other closely related compounds. Other unidentified vasodilator substances may also be present. The susceptibility of red blood cells to mechanical trauma has been emphasized. Need for critical evaluation of data from experiments employing the extra corporal circulation of blood had been pointed out.

We wish to express our thanks to Drs. Mika Hayano and W. K. Jordan for their advice, suggestions and the spectrometric analyses (W. K. J.).

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## ACTION OF TETRAETHYLAMMONIUM ON PRESSOR RESPONSE TO ASPHYXIA<sup>1</sup>

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**I**N A study of the action of the tetraethylammonium ion (TEA) on various autonomic cardiovascular reflexes it was found that blockade of ganglia with this drug failed to prevent the pressor response to asphyxia in the dog (1,2). In an attempt to explain this phenomenon, a number of possible mechanisms were considered.

First it was assumed that the adrenal medulla, though innervated cholinergically, and homologous embryologically with sympathetic ganglia, might escape the blocking action of TEA. This possibility would be more likely if the sympathetic fibers to the adrenal terminated intracellularly, but a careful study of the adrenals in many mammalian species including the dog failed to reveal such endings (MacFarland and Davenport, 3). Experiments were conducted, nonetheless, to determine the sensitivity of the adrenal innervation to TEA.

Second, it was considered possible that increased muscular and respiratory activity during exposure to asphyxia could increase venous return and cardiac output sufficiently to elevate arterial pressure in spite of blockade of autonomic vasoconstrictor and cardio-accelerator pathways.

Third, it was supposed that a direct action of hypoxia or hypercapnia on ganglion cells and the adrenal medulla, as demonstrated by Bülbring, Burn and De Elío (4), might provoke a discharge not vulnerable to the action of synaptic inhibitors. It had already been shown that the carotid chemoreceptors, which react pharmacologically much like ganglion cells, continue to respond to hypoxia and cyanide in the presence of a high concentration of TEA (5).

Finally, since pressor and cardio-accelerator pathways invulnerable to TEA have been shown to exist in the dog (6,7), it was considered possible that asphyxia might excite an auxiliary vasomotor system not ordinarily involved in the cardiovascular response to less intense stimuli.

The present report presents the results of experiments designed to elucidate the nature of the pressor response in several species.

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<sup>2</sup> Adapted from a thesis submitted by W. A. F. to the faculty of the Horace Rackham School of Graduate Studies of the University of Michigan in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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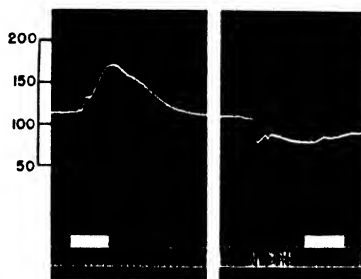
## RESULTS

*Dog*

*Action of TEA on the adrenal medulla.* In 2 dogs under thiopental-barbital anesthesia the left sympathetic chain was exposed at the level of origin of the splanchnic nerves. The intestines, spleen, and pancreas were removed to eliminate any possibility that stimuli applied to the splanchnic nerve could initiate a pressor response due to excitation of post-ganglionic vasoconstrictor fibers to the splanchnic vascular bed. Arterial pressure was recorded with a mercury manometer, and stimuli from an Electrodyne stimulator were applied at intervals before and during continuous infusion of TEA.

Stimulation of the splanchnic nerve caused a rise of pressure due to liberation of epinephrine from the adrenal. During infusion of TEA at a rate of 20 mg/kg/hour, the pressor response was abolished (fig. 1). It was concluded that the cholinergic innervation of the adrenal medulla is blocked by TEA in the dog, confirming the results obtained in the cat by Morrison and Farrar (8).

Fig. 1. EXPERIMENT 11/10/47; dog 12.5 kg., thiopental-barbital anesthesia; intestine, spleen and pancreas removed. *Tracings:* arterial pressure, signal, time in 10 sec.; *left:* stimulation of splanchnic nerve before TEA; *right:* TEA infusion (20 mg/kg/hr.) begun and priming dose at first signal, repeat stimulation of nerve.



In 4 additional experiments, the role of the adrenal medulla was tested by recording the response of arterial pressure to asphyxia before and after removal of the adrenals. Asphyxia was induced by rebreathing from a small closed system. Continuous infusion of TEA failed to block the asphyxial pressure rise, and the additional procedure of bilateral adrenalectomy also failed to prevent the response in 3 of the 4 experiments. It was concluded that while the adrenals may contribute to the asphyxial pressure rise in the untreated animal, they are not essential in the mediation of that response which persists after TEA.

*Role of increased skeletal muscle activity in the asphyxial pressure rise.* To test the possibility that the pressor response persisting after administration of TEA could result from the increased venous return and cardiac output caused by vigorous respiratory efforts, the effect of curare alkaloids ('Intocostrin' and D-tubocurarine) on the response was investigated in 3 dogs. In these experiments the curare preparations were administered intravenously in repeated doses as required to paralyze completely the diaphragm and intercostal muscles. Artificial respiration was instituted by means of a Starling pump, and asphyxia was produced by stopping the pump. Asphyxia caused a rise of pressure in the curarized animals, and the rise was not prevented by TEA.

In 4 experiments, the action of Dibenamine and of  $\alpha$ -naphthylmethylethyl- $\beta$  bromoethylamine hydrobromide (SY-28) on the asphyxial pressure rise was tested. Though these agents do not prevent the increased respiratory activity during asphyxia, the pressure rise was completely prevented. It was concluded that skeletal muscular activity contributed little to the asphyxial reaction.

*Role of direct asphyxial stimulation of adrenal medulla and ganglion cells.* This possibility was tested by the simple expedient of administering the asphyxial stimulus to the head while maintaining an adequate respiratory exchange in the trunk. In 2 experiments the head was perfused by way of the brachiocephalic artery and superior vena cava with heparinized blood from a donor animal. Both animals were anesthetized with  $\alpha$ -chloralose. Asphyxia in the donor dog (and the perfused head) was produced by rebreathing from a small bag, and changes of perfusion pressure were minimized by means of a pressure stabilizer attached to the carotid arteries of the donor.

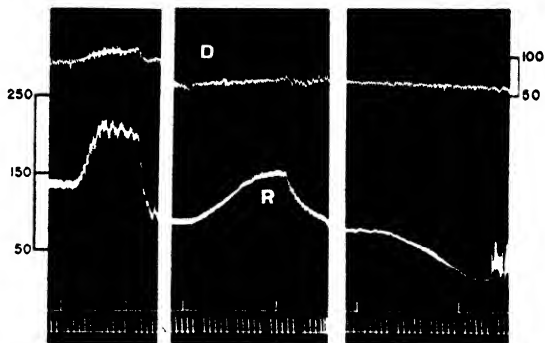


Fig. 2. EXPERIMENT 12/9/47; chloralose anesthesia; head perfusion. Tracings: arterial pressure of donor dog (*D*), pressure of recipient dog (*R*), signal, time; first segment: asphyxia of donor dog (and head of recipient); second segment: same during infusion of TEA in trunk of recipient; third segment: asphyxia of trunk of recipient dog during TEA infusion (head remains oxygenated).

Asphyxia of the perfused head caused a rise of pressure in the trunk which was diminished but not abolished by TEA (fig. 2).

In 3 additional experiments head asphyxia was produced by occlusion of the brachiocephalic and left subclavian arteries. Artificial respiration was maintained throughout. The heart was denervated by section of the vagi and the upper thoracic ganglia. Occlusion of the cephalic arterial supply caused a great increase of arterial pressure and presumably a liberation of epinephrine, as indicated by an increase of heart rate. Administration of TEA diminished but did not abolish the pressure rise, but usually eliminated the heart rate response to cephalic asphyxia (fig. 3). General asphyxia induced by arrest of the respiration pump in the same animals caused a moderate elevation of pressure and a greater post-asphyxial rise, neither of which were prevented by TEA (fig. 3).

*Cardiovascular pathways not blocked by TEA.* Some evidence has already been presented to demonstrate that cardio-accelerator and inotropic impulses to the

ventricles can be initiated by cephalic asphyxia in the dog, and that such impulses are interrupted by section of the preganglionic rami of the upper thoracic ganglia but not by TEA (6). To extend these observations to vasomotor pathways, cross-circulation experiments were devised. In 2 experiments one hind leg of a recipient dog was perfused by way of the femoral artery and vein from a heparinized donor animal. Blood flow in the perfused member was recorded with a differential manometer (9). Collateral connections to the leg were essentially eliminated by ligation of the aorta and vena cava just above their bifurcations. The leg was thus connected to the trunk by way of intact nerves, but was subjected to a stable perfusion pressure and protected against any significant admixture of blood from the recipient's circulation. Asphyxia was induced in the recipient animal by rebreathing, and the flow in the perfused extremity recorded as an index of neurogenic vasomotor activity.

Asphyxia of the recipient animal caused an increase of arterial pressure accompanied by a decreased blood flow in the leg, indicating the active participation of

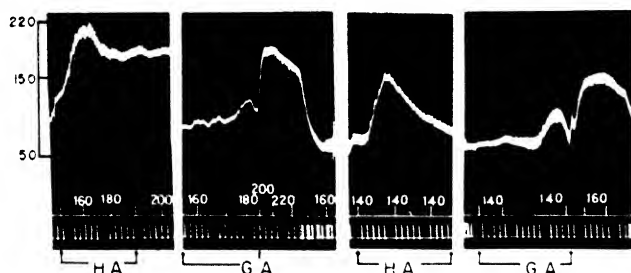


Fig. 3. EXPERIMENT 1/11/48; dog 11 kg., chloralose anesthesia; chest open, heart denervated. Tracings: as in figure 1, numbers refer to heart rate; first segment: head asphyxia (H.A.) induced by occlusion of vessels; second segment: general asphyxia (G.A.) induced by gas mixture; third and fourth segments: same procedures during infusion of TEA, 40 mg/kg/hr.

vasoconstrictor nerve impulses. After administration of TEA to the recipient animal, the vasoconstrictor response to asphyxia was delayed and reduced, but never interrupted.

In an additional experiment, perfusion of the leg was maintained by means of a constant output pump supplied from a reservoir connected to a carotid artery. Perfusion pressure between the pump and the leg was recorded as a measure of the resistance of the perfused member. An additional reservoir of blood attached to the contralateral femoral artery served as a buffer space. Just before the induction of asphyxia, the perfusion reservoir was filled with oxygenated blood, and its connection with the carotid clamped. After a brief control period, asphyxia was induced by rebreathing. Vasoconstriction occurring in the perfused leg could not be prevented by TEA, but was abolished by SY-28 (fig. 4).

In a similar experiment the left kidney was perfused from a reservoir of arterial blood under constant pressure. A rhythmic occlusion device established a pulsatile pressure, and renal flow was recorded with a differential manometer. Asphyxia of the animal caused renal vasoconstriction which could not be prevented by TEA.

### *Cat*

In 4 experiments on cats, the pressor response to asphyxia, produced by re-breathing, was recorded before and after administration of TEA. In 2 experiments the response was prevented by TEA. In the other 2 animals, the response was greatly diminished but not abolished by TEA. During asphyxia in these animals very vigorous respiratory and muscular movements occurred. When motor activity was eliminated by the administration of D-tubocurarine, the residual pressor activity was abolished, although this drug alone did not prevent a rise of pressure. The response to cephalic asphyxia, produced by occlusion of the brachiocephalic and left subclavian arteries, was likewise prevented by TEA and D-tubocurarine.

### *Monkey*

Three experiments on rhesus monkeys yielded results similar to those obtained from cats.

### *Rabbit*

Four experiments were performed on rabbits. In 2 animals the control pressor response to asphyxia was poor, and was reduced by TEA. In the other 2 animals the pressor response to asphyxia induced by rebreathing or by occlusion of the cephalic vessels was not significantly altered by large doses of TEA and curare.

### DISCUSSION

The rise of pressure which occurs during respiration of an asphyxial gas mixture probably represents the combined activation of arteriolar constrictor, venoconstrictor, and positive chronotropic and inotropic cardiac mechanisms, together with an increased venous return resulting from increased motor activity. The major efferent outflow must be by way of sympathetic pathways, but the above experiments demonstrate that interruption of ganglionic transmission by means of TEA fails to prevent the activation of pressor mechanisms in the dog. This failure cannot be due to a lack of effect of TEA on the adrenal medullary neurosecretory mechanism, which is readily blocked by the drug; nor can it be due primarily to a direct effect of asphyxia on the medulla and ganglion cells, since the response to cephalic asphyxia also persists in the presence of TEA. It seems likely, then, that an auxiliary pressor pathway resistant to the blocking action of the drug exists in the dog.

As indirect evidence in support of the concept of an auxiliary pressor system concerned with the mediation of the cardiovascular response to asphyxia, we may cite the experiments described in the preceding paper (7). Stimulation of the cord excites pressor pathways which are interrupted by TEA in the cat and monkey, in which species the asphyxial pressure rise is also blocked, but not in the rabbit and dog, in which the asphyxial response is not prevented.

Direct evidence can be drawn from the experiments described by Pardo *et al.* (6). Though TEA was shown to block the cardio-accelerator response to occlusion of the carotid arteries, it did not block the cardiac effects of cephalic asphyxia. Section of thoracic sympathetic preganglionic rami, however, completely insulated the heart against the cephalic asphyxial stimulus. In the dog, at least, sympathetic

pathways not interrupted by TEA were thus shown to carry the asphyxial efferent outflow.

The cross-circulation experiments of the present report likewise demonstrate the existence of neurogenic vasomotor paths which can be activated by asphyxia in the presence of concentrations of TEA which adequately block other cardiovascular autonomic channels.

These observations still do not indicate the nature of the auxiliary vasomotor pathway. Whether the cardiovascular impulses aroused by asphyxia course without synaptic interruption to the heart and vessels, or are interrupted by a qualitatively

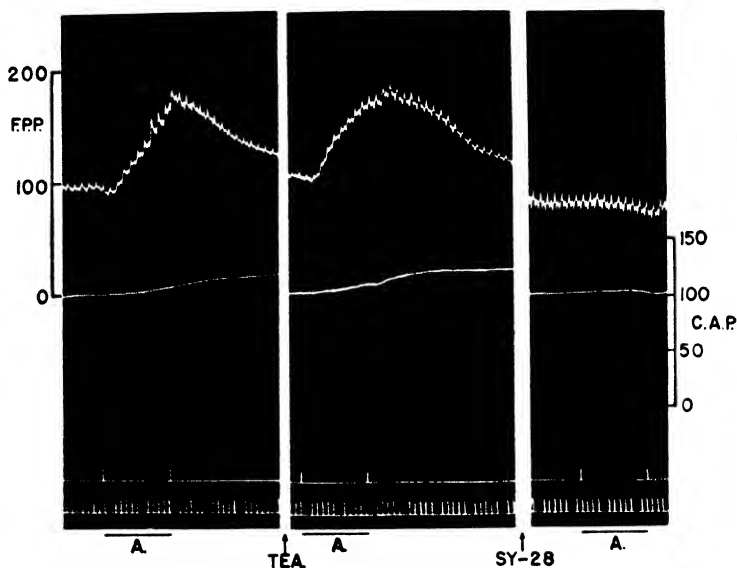


Fig. 4. EXPERIMENT 6/24/50; dog 16 kg., thiopental-barbital anesthesia; femoral perfusion. Tracings: perfusion pressure from constant output pump at 60 cc/min., systemic arterial pressure (pressure stabilizer attached to contralateral femoral artery); signal, time. A: asphyxia produced by rebreathing; TEA: 2 doses of TEA totaling 25 mg/kg.; SY-28: 1.3 mg/kg. SY-28.

different type of ganglionic synapse cannot be decided with the data now available. The observations of Shaw *et al.* (10), who postulated two kinds of sympathetic ganglion cells mediating vasodilator and vasoconstrictor impulses, might be considered to support the latter hypothesis, but their data cannot in our opinion be considered as incontrovertible proof of the existence of two kinds of ganglia.

Whether asphyxial pressor mechanisms are mediated solely through medullary centers, or supported in part by the activation of higher, perhaps hypothalamic, cell stations cannot at present be determined. If we may conclude that there exists in the dog an auxiliary, independent, emergency pressor mechanism, it would not be surprising if the central organization of that system were essentially independent of the medullary centers governing the ordinary barostatic reflexes.



## SUMMARY

The pressor response to asphyxia is blocked by TEA in the cat and monkey, but not in the rabbit and dog. Failure to prevent the pressure rise in the dog is not due to anoxic stimulation of ganglion cells and the adrenal medulla, nor to failure of TEA to block the innervation of the adrenal, nor to a major participation of skeletal muscle activity, but is probably due to the intervention of an auxiliary pressor mechanism which cannot be interrupted by the ganglionic blocking agent.

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# RELATION OF MUSCULAR FATIGUE IN THE ADRENALECTOMIZED DOG TO INADEQUATE CIRCULATORY ADJUSTMENT

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ADRENALECTOMIZED rats (salt-treated, in electrolyte balance) cannot sustain forced work (e.g. swimming) for more than a brief period of 20 to 30 minutes. The normal controls can swim without interruption for many hours (1,2). This extreme fatigability has been attributed to some undefined metabolic disturbance in the neuromuscular apparatus which follows deficiency of the C<sub>11</sub> steroids (3). We have previously demonstrated that in contrast to the fatigability *in vivo*, muscle strips removed from adrenalectomized animals and stimulated *in vitro* do not differ from normal muscle in work performance. *In vitro*, such muscle strips (diaphragm and abdominal muscle) are, of course, kept in an oxygenated, buffered medium containing glucose as a nutrient. *In vivo* a working muscle requires that the flow of blood through it be adjusted to provide adequate oxygenation and transfer of metabolites. We, therefore, concluded from the above data that the increased fatigability exhibited by the adrenalectomized muscle, tested *in vivo*, may be due to an inadequate adjustment of the circulation to the metabolic needs of extra work.

Accordingly, we have investigated the behavior of the blood pressure during work performance in the adrenalectomized animal in normal electrolyte balance, in order to determine whether major circulatory changes occur prior to the onset of fatigue.

## METHODS

Four dogs weighing 7 to 19 kg. were bilaterally adrenalectomized and maintained on daily intramuscular injections of 2 mg. of desoxycorticosterone acetate in oil. The regular diet (commercial dog food) was supplemented by milk and eggs (by stomach tube if necessary) to insure adequate nutrition. The animals were used for study 5 to 14 days postoperatively and appeared to be in good general condition. Blood chloride and glucose levels were determined prior to each experiment. Two dogs were employed as normal controls.

The experiments were performed under sodium pentobarbital anesthesia. A

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<sup>2</sup> The department is in part supported by the Michael Reese Research Foundation.

direct writing mercury manometer was inserted into the left femoral artery. The connecting tubing etc. was filled with heparinized saline. The right hind limb was fixed by means of a clamp at the level of the lower end of the femur. The tendon of the gastrocnemius was detached from its insertion to the heel. A weight was fixed to the freed tendon by a suture and suspended over a pulley so that the muscle would contract against it. For animals over 10-kg. body weight a 2-kg. wt. was used while a 1-kg. wt. was used for the smaller animals. The weight was also attached to the writing lever of a kymograph by a thread so that muscle contractions could be recorded. Needle electrodes were inserted into the tendon and into the belly of the gastroc-

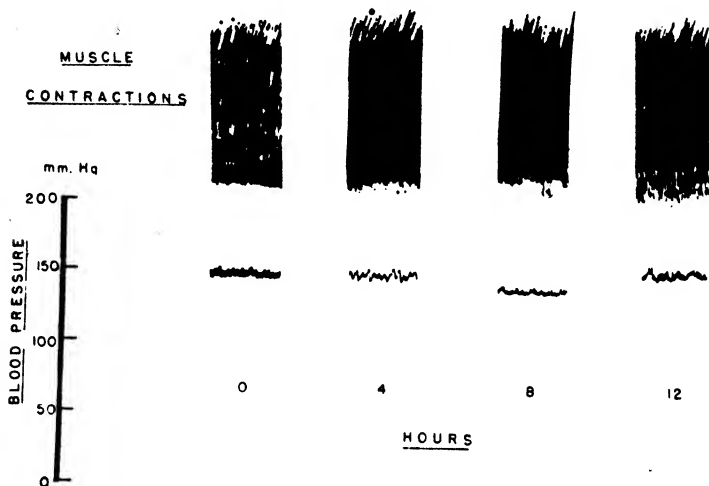


Fig. 1. NORMAL DOG, wt. 19 kg.; right gastrocnemius muscle stimulated in situ at 3/sec.; 2-kg. load. Note the excellent circulatory adaptation to uninterrupted stimulation of the muscle for 12 hours, as shown by maintenance of initial blood pressure level. No muscular fatigue under conditions shown.

nemius close to its origin. Electrical impulses were provided to these electrodes at the rate of 3/second from a thyratron tube stimulator.

#### RESULTS

Two normal dogs were observed for 6 and 12 hours respectively. In both cases, there was no appreciable change in either the blood pressure or the work performance despite uninterrupted stimulation (fig. 1). Both animals were then sacrificed.

In the 4 adrenalectomized dogs, blood chlorides were found to be 605 to 650 mg. per cent (as NaCl) and glucose 60 to 87 mg. per cent i.e. essentially normal values. In 3 of the dogs initial blood pressures were 95, 110, 150 mm. Hg respectively. A consistent pattern of blood pressure and work performances was seen in these animals. Both blood pressure and muscle contractions were maintained for a time at the initial levels. The blood pressure then began to fall and continued steadily downward until the death of the animal. Diminution of muscle contractions appeared only after the blood pressure had fallen to levels of about 60 to 75 mm. Hg (fig. 2).

Table 1 outlines the timing of these events. In all 3 animals falling blood pressures preceded any evidence of fatigue by an appreciable length of time (10-45 minutes).

The fourth adrenalectomized dog had an initial blood pressure of 70 mm. Hg. In this case, stimulation resulted in an immediate and progressive fall in blood pressure. Muscle fatigue was evident almost from the first contraction. Death occurred after 60 minutes.

Figure 3 is a summary graph of the data obtained in all our animals, in which the muscle performance is plotted against the simultaneously recorded blood pressure. It can be seen that no significant decline in muscular contractile power occurs at

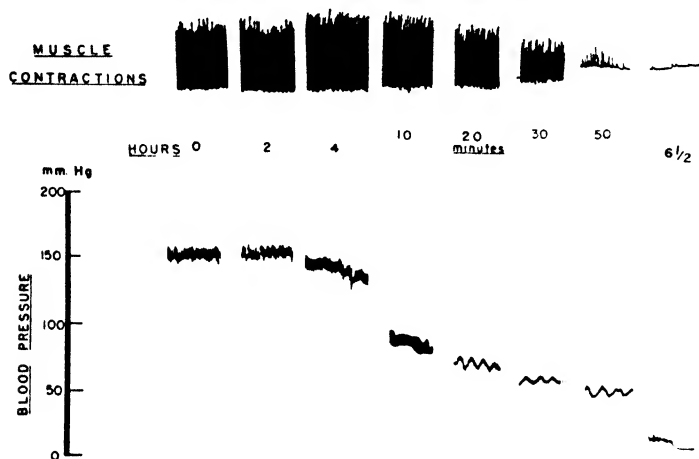


Fig. 2. ADRENALECTOMIZED DOG, wt. 7.6 kg.; right gastrocnemius muscle stimulated in situ at 3/sec.; 1-kg. load. Conditions of stimulation the same as shown in figure 1. Note that the adrenalectomized dog (despite adequate DOCA therapy) adapts poorly to demands of muscle work. Fall in blood pressure precedes muscular fatigue by an appreciable period. The animal dies as a result of circulatory failure induced by the attempt to adapt to stress.

blood pressures above 70 to 75 mm. Hg. Below these figures there is a direct relationship between blood pressure and height of contraction.

Temporary restoration of adequate blood pressure levels (by transfusion or pressor drugs) leads immediately to a partial or complete restoration of the initial height of contraction.

#### DISCUSSION

Our previous work indicated that muscles of adrenalectomized animals did not exhibit fatigue, *in vitro*, if oxygen and nutrients were continually supplied. The results of the present study show a relation between blood supply and fatigability in the intact adrenalectomized animal. Although the blood pressure is not per se a measure of cardiac output or rate of blood flow to the muscles, its progressive fall serves here as a consistent indicator of a disturbed and failing circulatory status of the adrenalectomized animal. This failure precedes and finally leads to fatigue of muscles working under conditions of inadequate supply. As long as blood pressure is maintained, work

performance is unfailing and effective. In the normal control this pattern is observed for many hours. In the adrenalectomized animal, the initial behavior is 'normal'

TABLE I

CONDITION OF ANIMALS	TIME FROM BEGINNING OF STIMULATION TO:		
	Onset of B.P. Fall	Onset of Fatigue	Death
	<i>min.</i>	<i>min.</i>	<i>min.</i>
Adrenalectomized	48	65	79
	280	290	300
	235	280	330
Normal	Blood pressure maintained until sacrificed No fatigue manifested (6 and 12 hours)		

but with time there is a deterioration of those adjustments of the circulation which maintain adequate supply of blood to the tissues.

The normal animal when presented with a work demand experiences a variety of reflex hemodynamic adjustments (local vasodilation, splanchnic constriction,

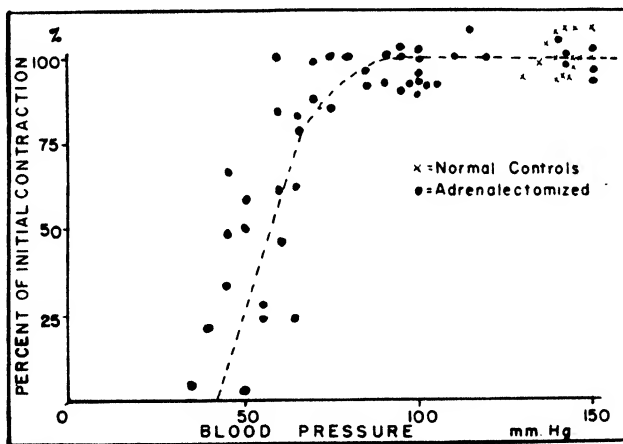


Fig. 3. SUMMARY GRAPH showing direct relationship between height of arterial blood pressure (between 30 and 75 mm. Hg) and degree of muscular contractile power. This relationship obtains equally well in normal animals as indicated by work of Schweitzer (4).

cardiac acceleration etc.). These serve to divert blood under adequate pressure to the working musculature of the body and represent a complex adjustment of the entire organism. In the adrenalectomized animal sustained work performance appears to elicit a similar pattern of adjustments of the neuro-circulatory systems of regulation so that the increased demands of working muscle are met effectively but only for a short time. These minute by minute adjustments soon begin to break down and a progressive fall in blood pressure is seen. Under the conditions of our experiments, this progressive hypotension is continuous and leads to death of the animal. At some

point this failing circulation reaches a critical level; the blood supply of the working muscles becomes insufficient and fatigue is manifested.

Prior to the application of the work demand, the adrenalectomized animal was maintaining itself reasonably well under more or less stress-free conditions. With the onset of stimulation, a process of maladaptation of the neuro-circulatory system was instituted which not only failed to meet the demands of work at one point in a declining curve of circulatory efficiency, but continued irreversibly until death of the animal.

It would seem, therefore, that the poor work performance of the adrenalectomized animal is a purely secondary result of a failure in circulatory adjustments. Our experiments suggest that the great variety of stresses which have been applied to adrenalectomized animals (cold, heat, injury, drugs etc.) may constitute similar demands for proper neuro-circulatory adjustments, which in the absence of C-11 oxy-steroids are distorted and lead to a similar breakdown.

#### SUMMARY AND CONCLUSIONS

Simultaneous work performance (gastrocnemius muscle) and blood pressure studies were made in normal and DOC-treated adrenalectomized dogs. Normal dogs maintained work performance and blood pressure for 12 hours of continuous stimulation, at their initial levels. Adrenalectomized dogs maintained normal work performance and blood pressure only for a short time. Within 1 to 4 hours a progressive and finally fatal fall in blood pressure occurred. The blood pressure fall preceded the signs of muscular fatigue in all cases. The failing work performance of adrenalectomized animals is a result of circulatory maladjustment to the demands of work. The concept of a maladaptation of the neuro-circulatory systems of regulation due to C-11 oxy-steroid lack is discussed in relation to muscle fatigue, and to the general sensitivity of the adrenalectomized animal to stress.

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# CIRCULATORY CHANGES DURING PROCESS OF AROUSAL IN THE HIBERNATING HAMSTER<sup>1</sup>

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THE golden hamster, *Mesocricetus auratus*, when placed in a sufficiently cold environment, enters, after a variable period, into a state of hibernation characterized by immobility and a profound lowering of body temperature and metabolism (1). When such an animal is handled or otherwise disturbed, there is a gradual increase in skeletal muscular activity, heart rate and oxygen consumption, with the result that the previously torpid animal is fully awake and active within 3 hours and has regained its normal body temperature of about 37° C. (1, 2).

The present investigation deals primarily with the changes that occur in the circulatory system of the hamster during arousal from hibernation. A consideration of these and previous results, however, has led us to formulate an hypothesis of the mechanism of arousal which will be presented in the discussion.

## METHODS

Adult hamsters were kept in a cold room at 5°C.  $\pm$  2° until they had entered hibernation. Most of the studies were carried out with the hamsters continuously in the cold room. In some preliminary experiments the animals were removed from the cold room and quickly transferred to an insulated refrigerated box maintained at the same temperature. Our results are based upon observations made on approximately 100 animals.

Electrocardiograms were usually obtained by inserting fine copper wire electrodes subcutaneously in the left axilla and back, recordings being made with a Grass ink-writing oscillograph. An oesophageal lead was used in order to disclose good P waves.

Body temperature of the hamsters was measured with iron-constantin thermocouples, one being placed deeply in a cheek pouch and one in the rectum (see Lyman (1) for details). Temperatures of the hamster and the environment were then recorded on a Micromax thermo-electric recorder (Leeds and Northrup) calibrated to an accuracy of  $\pm 0.5^\circ\text{C}$ .

It has previously been shown (1-4) that in animals recovering from the hibernating state the rectal temperature lags markedly behind that of the fore part of the body.

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Accordingly, in equating the various functions of the heart with the temperature, the cheek pouch temperature was used since this has been demonstrated (2, 3) to be essentially the same as that of the heart.

Blood pressures were obtained from a carotid artery, using either a mercury or a condenser manometer. The vagi were exposed in the neck and stimulated through shielded silver wire electrodes. A Grass model 3 stimulator was used. Drugs were given as follows: epinephrine (1 mg/kg.) directly into the heart; veratrosine (1 mg/kg.) intravenously or intra-arterially<sup>3</sup>; atropine (1 mg/kg.) intraperitoneally. The test for complete atropinization of the animal was the abolition of reflex vagal slowing of the heart from anoxia.

No anesthesia was necessary, as the hamsters were either painlessly killed or released when they had aroused sufficiently to show signs of discomfort.

### RESULTS

*Relation of Heart Rate to Temperature.* The heart of the hamster in deep hibernation beats very slowly, though the rates vary from animal to animal even at the same temperature. Table 1 shows rates recorded at random from 21 animals hiber-

TABLE 1. HEART RATES OF 21 HIBERNATING HAMSTERS EXAMINED AT RANDOM IN COLD ROOM AT 5°C.

HEART RATE/MIN.	NO. OF ANIMALS	HEART RATE/MIN.	NO. OF ANIMALS	HEART RATE/MIN.	NO. OF ANIMALS
21	1	15	1	8	5
19	1	12	2	7	2
18	1	11	1	6	1
16	1	9	4	4	1

nating in the cold room. The environmental temperature was 5°C. and the body temperatures of the hamsters were the same or less than a degree above this.

The manipulations necessary to prepare the hamster for taking records invariably initiated the process of arousal from hibernation. As the animal roused and its body temperature increased, the heart rate also increased, at first slowly and then more quickly and eventually linearly (fig. 1). A plot of the logarithm of the heart rate against the reciprocal of the absolute temperature shows that the function is not a straight line. In figure 2A, it is seen that as the temperature increased the heart rate gradually fell below the values which would be predicted if the relationship were a logarithmic one. Therefore, these data do not fit the Arrhenius equation which describes simple physico-chemical processes.

That this gradual decrease in the Arrhenius constant  $\mu$  was intrinsic to the heart itself, rather than due to the influence of the vagus on the heart rate, was shown by records of the rates of 3 completely atropinized awakening hamsters. A comparison of these results (fig. 2B) with those from normal animals reveals that the curves are similar in shape. Thus, there is no evidence that failure to fit the

<sup>3</sup> We are extremely indebted to Dr. Otto Kraye of the Department of Pharmacology, Harvard Medical School, for generously supplying us with veratrosine.



Arrhenius equation was due to a tonic slowing action of the vagus. Nor was this lack of vagal action due to an inability to function at low temperatures, since it was found that the heart of the hamster could be slowed by stimulating the peripheral end of the severed right vagus when the animal's body temperature was as low as  $10^{\circ}\text{C}$ . On the other hand, the vagus did take effect in the last stages of the waking process, for then the heart showed periods of irregular bradycardia which could be abolished by atropine.

*Activity of Sympathetico-Adrenal System during Arousal.* The veratrum derivative *veratrosine* (veratramine glucoside) has been shown to have a sympathetolytic action which is confined to the pacemaker and prevents the cardio-accelerator action of both circulating epinephrine and probably the cardiac sympathetic nerves (5-7).

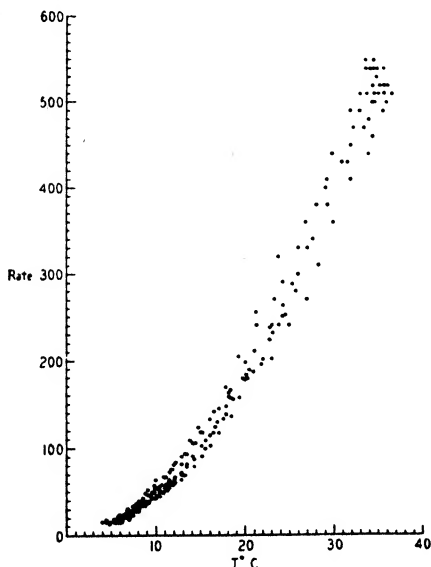


Fig. 1. COMPOSITE GRAPH of the heart rates/min. of 6 hamsters arousing from hibernation, plotted against their cheek pouch temperatures. Linear coordinates.

When administered to hamsters arousing from hibernation, veratrosine (1 mg./kg.) caused a slowing of the heart. This impaired the process of arousal as manifested by a slowing in the rate of increase of body temperature (fig. 3). With smaller doses which had a briefer action, the cardio-accelerator mechanisms returned the heart to beating at its maximal rate when the effects of the drug had worn off, and the rapid increase in body temperature was resumed.

These results indicate that during arousal the heart of the hamster is being driven at maximal rates by the sympathetico-adrenal system, and indeed that these maximal rates are prerequisite for the most efficient and rapid return to the normal homeothermic state.

This conclusion is further supported by the fact that during arousal the heart did not accelerate further in response to injected epinephrine.

*Pacemaker Mechanisms and Velocity of Conduction of the Cardiac Impulse.* A-V dissociation has been frequently described in hibernating animals at low tempera-

tures (8-10). In non-hibernating animals the body temperatures of which had been lowered artificially, auricular fibrillation, abnormally slow conduction, and 2:1 and 3:1 A-V blocks have been reported (11, 12). If the electrocardiogram of hamsters was taken early enough in the process of arousal, A-V dissociation was occasionally seen and it is probable that this situation may obtain in deep hibernation. However, many animals, by the time they had been prepared for recording, showed no dysrhythmias. Figure 4 shows the electrocardiogram of an animal in which complete A-V dissociation was seen initially. Figure 4A shows the dissociation of P waves and QRS complexes, each occurring at irregular though similar rates (15/min.). Within a few minutes (fig. 4B), the rhythm had become regular at 15/minute, with the P wave now regularly preceding the QRS complex. Thenceforth, the rate accelerated

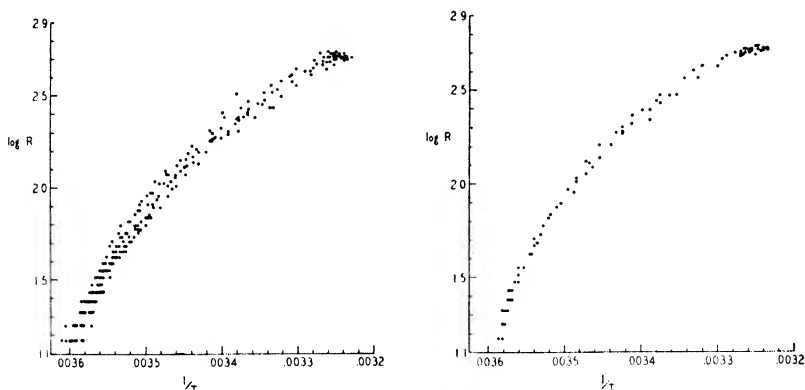


Fig. 2A (left). DATA OF FIGURE 1 plotted to test for an exponential relationship. Ordinate: logarithm of the heart rate/min.; abscissa: reciprocal of the absolute body temperature. See text.

Fig. 2B (right). A SIMILAR PLOT of the heart rates and body temperatures of 3 completely atropinized hamsters. See text for discussion.

in the way that has been described. A point of interest is that the ventricular complexes of figures 4A and 4B are quite similar, indicating that the rhythm in figure 4A was not only idioventricular but originated at the A-V node. It is to be noted also that the R wave is notched. This may be interpreted to represent an impairment in the intraventricular conduction system, which incidentally disappeared as warming progressed.

In non-hibernating animals which have been artificially cooled, the rate of conduction of the cardiac impulse has been found to vary linearly with the heart rate (11-14). In arousing hamsters, the rate of conduction over the auricle and through the A-V node (as measured by the reciprocal of the P-R interval) also varied linearly with heart rate, while the duration of the QRS complex shortened markedly as heart rate increased. However, accurate measurements of the latter were precluded by the filtering condensers necessary for minimizing the muscle action potentials and slow base line shifts arising in the unanesthetized animal.

Another rather notable change that occurred in the electrocardiogram during arousal was an increase in the prominence of the T wave (fig. 5). This change probably

signifies the development of unequal rates of repolarization in the two ventricles. Later, when the heart was beating more rapidly (fig. 5, lowest record), it was seen that the P wave arose from a baseline displaced by the T wave. This signifies that the heart was beating so rapidly that another impulse must have been traversing the auricles before the ventricles were completely repolarized. The mechanisms of such a heart must be beautifully timed to give at such high rates a regular rhythm without dropped ventricular beats.

The anatomy of the heart of the hamster has been described by Walls (15). He finds that the heart shows the following peculiarities: the sino-atrial node itself contains Purkinje fibers, which are otherwise absent in the atria; the atrio-ventricular node contains fibers which approach the Purkinje type in size and general appearance; and finally there is no Purkinje tissue in the right ventricle and only a limited amount in the left. It is surprising that a heart which normally functions at such high rates contains so little specialized conducting tissue.

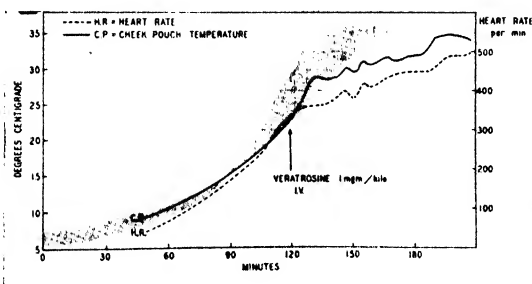


Fig. 3. EFFECT OF a pharmacological slowing of heart rate on the process of arousal. *Shaded area* indicates the extremes of variation of cheek pouch temperature of eight control hamsters during arousal from hibernation. The time at which the temperature reached  $15^{\circ}\text{C}$ . has been arbitrarily set at 90 mins. in each case for comparison. *Heavy black line* shows the cheek pouch temperature and *broken line* the heart rate of a typical hamster whose heart was slowed by treatment with veratrosine (at arrow). For further discussion, see text.

*Changes in Blood Pressure during Arousal.* It has previously been observed (1) that the paws of the hibernating hamster are bright pink in color, and that after the process of arousal has been initiated the skin color becomes very pale. In addition, it was shown (2) that there exists in the awakening hamster a differential vasoconstriction of the deeper tissues of the body, with blood flow to the anterior part unimpeded while that to the posterior part is restricted. This differential vasoconstriction apparently accounts for the large temperature gradient that exists between the fore and hind portions of the animal. In view of these peripheral changes during arousal, changes in blood pressure were also studied.

It was, of course, impossible with present technics to measure the blood pressure of the hamster *during* hibernation. By the time a cannula had been inserted in a carotid artery and arrangements made for recording, the waking process was well under way. Nevertheless, it is apparent that blood pressure was initially low (fig. 6). As the process of arousal continued, the heart rate increased at first relatively slowly, while the blood pressure climbed at a much faster rate. After this initial steep rise, the pressure rose more slowly until a maximum was reached.

It was observed that the highest value of blood pressure was reached long before the heart was beating at its maximum rate. In all animals it was also noted that only when the blood pressure was approaching or had attained its maximal value did the greatest increments of heart rate and body temperature occur. It can also be seen that pulse pressure increased transiently and then declined as the heart rate increased further.

In general, the blood pressure of arousing animals tended to be somewhat lower than that of non-hibernating hamsters under light nembutal anesthesia whose mean pressure was found to be 120 to 170 mm. Hg at rates of 300 to 400/minute.

Thus, the series of events during arousal would seem to be as follows: the waking stimulus initiates a differential and cutaneous vasoconstriction as the heart begins to beat faster. The increasing peripheral resistance and pulse rate serve to bring the blood pressure quickly to maximal levels at least in the fore part of the body. After this, the process of heat production and distribution proceeds at much more rapid rates and the animal soon returns to its normal homeothermic state.

#### DISCUSSION

The heart rate of the awakening hamster increases at first slowly and then at a temperature of about  $13^{\circ}\text{C}$ .<sup>4</sup> accelerates more rapidly, above  $20^{\circ}\text{C}$ . varying approxi-

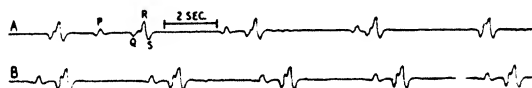


Fig. 4. INK WRITER RECORD of the electrocardiogram of a hamster early during the process of arousal. Letters indicate components of electrocardiogram. A. showing A-V dissociation early during arousal; B. a few minutes later a normal rhythm has been attained.

mately linearly with the temperature. During this latter rise, the rate falls below that which would be expected if the function were a logarithmic one, even though, as has been shown, the vagus is inactive and the heart is apparently being driven at maximal rates by the sympathetico-adrenal system.

Many of the data in the literature reveal a similar approximately linear relationship between heart rate and temperature in warm-blooded animals. However, in frogs, Barcroft and Izquierdo (16) obtained both linear and exponential curves, while Carter (17) obtained exponential curves from winter frogs and linear curves from summer frogs. Similarly, Endres *et al.* (9) obtained exponential curves from a marmot awakening from hibernation. However, the latter investigators placed thermocouples in the heart itself, which might have disturbed conduction, and the temperature range covered was small.

The fact that the rate of change of the slope of the curve of heart rate plotted against temperature (fig. 1) shows a discontinuity at about  $13^{\circ}\text{C}$ . can perhaps be ascribed to an increased effectiveness of sympathetico-adrenal activity, since Gellhorn (18) has shown that epinephrine increases the temperature coefficient of the heart.

A review of the results presented in this paper and preceding ones (1, 2) discloses

<sup>4</sup> Analysis of the individual data from which figure 1 was plotted shows that in 5 of the 6 animals there was a discontinuity in the first derivative of rate as a function of temperature at about  $13^{\circ}\text{C}$ .

that as the hamster arouses from the hibernating state several phenomena occur. There is a sudden increase in the electrical activity of skeletal muscle. If A-V dissociation is present, it is abolished and the A-V rhythm becomes normal. The heart rate begins to increase, slowly at first, and then more quickly with a concurrent rapid increase in rate of conduction of the cardiac impulse. The cutaneous vessels constrict, as indicated by a sudden pallor of the paws, and the deeper vessels undergo a differential vasoconstriction which impedes the circulation to the posterior part of the body while leaving that to the anterior part unrestricted. Concomitantly, the blood pressure rises rapidly to near normal levels. Visible muscular movements and violent shivering begin to occur and the body temperature returns at an accelerating rate to normal levels as the oxygen consumption increases 30-fold or more. The

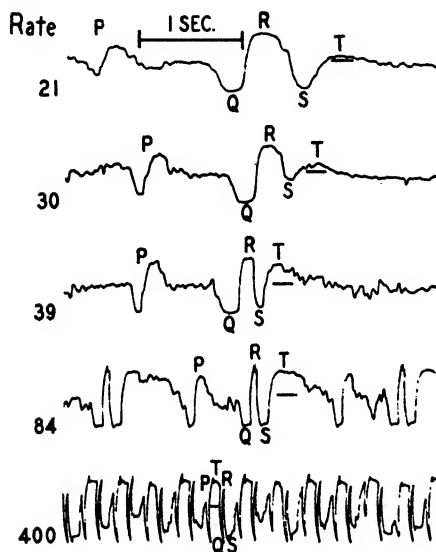


Fig. 5. INK WRITER RECORD of electrocardiogram of a hamster during arousal showing increasing rate, shortening of P-R interval and duration of QRS complex, and changes in T wave. Letters indicate components of electrocardiogram. Horizontal line under T wave in each case is isoelectric line. Minor irregularities due to muscle action potentials.

possibility of a mass discharge of the hypothalamus would most logically account for the appearance of these many coordinated autonomic and somatic phenomena.

Thus the hypothesis herein presented for the overall mechanism of the process of arousal from hibernation is that the waking stimulus causes a mass discharge from the hypothalamus which leads to the initiation of the sympathetic and somatic activities described above. This hypothesis is in agreement with the conclusion of Johnson (19) that during arousal in the ground squirrel the heat regulating mechanisms are brought irreversibly into activity. Furthermore, Johnson showed that mechanical stimulation of awakening ground squirrels could not hasten the process of arousal.

The hypothalamus has been implicated in the phenomenon of hibernation previously; Quincke (20) was perhaps the first to do so. Dubois (4) noted that "toward the anterior part of the aqueduct of Sylvius and at the sides of the floor of

the third ventricle" were centers necessary for awakening in the marmot (p. 175). Others (21, 22) have speculated that the sympathetico-adrenal system is involved in arousal from hibernation. Our results seem to furnish evidence for these views.

Merzbacher (23) many years ago pointed out that normal hibernation is a reversible state from which the animal can recover spontaneously. On the other hand, an artificially cooled hibernator will remain chilled and die if not warmed by an external source of heat. Thus, one or more changes must occur in the hamster exposed to cold which prepare it for a successful sojourn at, and return from, the low body temperature which obtains during hibernation.

Marès (24) came to the pertinent conclusion that the initial cause of hibernation was the ability of the nervous system to lose its specific sensitivity to external cold. We have again emphasized the probable role of the hypothalamus in the process of hibernation and arousal because the essential gaps in our knowledge of the hiberna-

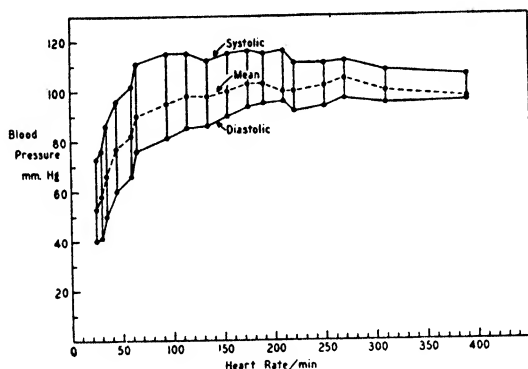


Fig. 6. SYSTOLIC, DIASTOLIC, and mean blood pressures of a typical arousing animal plotted against heart rate, showing that blood pressure reaches high values long before the heart is beating at its maximal rate. Pressures were obtained with a condenser manometer which electrically integrates systolic and diastolic pressures to give the mean pressure.

tion process as a whole seem to involve the mechanisms by which the thermoregulatory centers of these animals can be reversibly inactivated and restored to function.

#### SUMMARY

The golden hamster, *Mesocricetus auratus*, when kept in a cold environment eventually enters a state of hibernation characterized by immobility and a profound lowering of body temperature and metabolism. When the animal is disturbed, the waking process is initiated and the following physiological changes can be demonstrated: increase in heart rate, slow at first and later approximately linear with increase in body temperature; abolition of A-V dissociation, if present; increase in velocity of conduction of the cardiac impulse; increase in blood pressure, and, as previously reported (1, 2), cutaneous vasoconstriction, differential vasoconstriction between fore and hind parts of the body, activation of the somatic muscular system and great increase in oxygen consumption.

During arousal from hibernation, there is no evidence that the vagus is slowing the heart rate. On the contrary, there is evidence from the use of epinephrine and veratrosine that the heart is being driven at maximum rates for each body temperature by the sympathetico-adrenal system. All these phenomena can be correlated in the hypothesis that the process of arousal is essentially a mass activation of the centers of the hypothalamus which govern heat production and conservation and which give rise to maximal functional activity of the sympathetico-adrenal and somatic motor systems. It is pointed out that an understanding of the manner in which these centers are inactivated and activated will contribute greatly to our knowledge of the phenomenon of hibernation.

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# CIRCULATORY TRANSFER OF $P^{32}$ TO SKELETAL MUSCLES UNDER VARIOUS EXPERIMENTAL CONDITIONS<sup>1</sup>

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THE importance of an adequate circulation for the transfer of material to and from skeletal muscle is well recognized. The quantity of any diffusible substance which will enter a muscle within a given period of time is determined by a number of factors. These include the volume of blood flow, the relative concentration of the diffusible substance in the plasma and extracellular water, the area and permeability of the capillary bed and the intracellular uptake by the muscle fiber. The present studies represent an attempt to quantitatively measure the uptake of  $P^{32}$  by skeletal muscle under various experimental conditions. The conditions which were investigated were limited to those which permitted the gastrocnemius of one limb to serve as the experimental muscle and that of the contralateral limb as its control. The conditions of the experiments were arranged so that the principal variables were the volume of blood flow and the area and permeability of the capillary bed. The experiments to be reported were carried out on the gastrocnemius muscles of a total of 192 adult rats. The animals were unanesthetized unless otherwise indicated. The general plan was to make an intraperitoneal injection of 2 cc. of a solution containing approximately 0.4 millicuries of  $P^{32}$  in the form of phosphoric acid.<sup>2</sup> The animals were decapitated 30 seconds later and the gastrocnemii dissected out and weighed. In 4 experiments blood was collected at the time of decapitation, serum prepared and analyzed for  $P^{32}$  activity. Serum was also analyzed in a similar manner from 4 animals decapitated 240 seconds later and from 5 animals decapitated 960 seconds later. The muscles were ground up with 10 cc. of 5 per cent trichloroacetic acid; the acid filtrates were centrifuged, and the radioactivity of the liquid samples were measured in counts per second by means of a jacketed Geiger-Müller counter tube. This tube consists of a glass jacket surrounding the sensitive volume of the Geiger-Müller tube. It was assumed that the muscle was composed of 80 per cent water and a correction was made for the dilution due to this added volume of fluid. Care was taken to see that the activities of both samples from an animal were measured at approximately the same time. The total activity count in the experimental gastrocnemius was expressed as percentage of that in the contralateral control muscle. The relative activity was also calculated on a gram weight basis. A statistical analysis of the data was subsequently made in which the level of significance was calculated with the aid of Student's 't' table (1).

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<sup>2</sup> The  $P^{32}$  used in this investigation was obtained from the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.



A comparative study was made of activity counts in the right and left gastrocnemius muscles of 13 normal control animals. The effect of strong sustained muscular contraction on phosphate uptake was measured in experiments on 8 animals in which strong induction shocks were applied to the gastrocnemius muscle of one limb for the 30-second period of time between phosphate injection and decapitation. In other experiments on 10 animals the phosphate injections were made at the end of a 30-second period of muscle stimulation and the uptake of  $P^{32}$  was measured in the post-exercise period. The electrical stimulation was carried out on animals under light ether anesthesia. Studies were made on 10 animals in which one limb was immobilized for 5 days in mid-extension position with the aid of plaster bandages. Unilateral tenotomy was accomplished in 13 animals by sectioning the os calcis.

Denervation of the gastrocnemius muscle of one limb of a total of 59 animals was accomplished either by sectioning the sciatic nerve or by crushing the tibial nerve at its juncture with the peroneal. Either procedure produces complete denervation for a period of 14 days. However, with the latter procedure the onset of reinnervation occurs

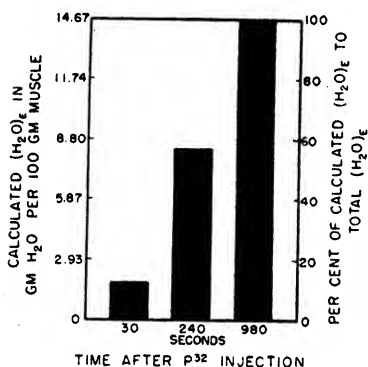


Fig. 1.  $P^{32}$  UPTAKE BY NORMAL MUSCLE at various times after intraperitoneal injection. Values are expressed in terms of calculated equilibria between serum and muscle extracellular water.

approximately at 14 days after denervations (2). Studies were carried out on groups of animals at 7, 10, 14, 21, 28 or 42 days after denervation. In studies carried out on animals for more than 14 days, denervation was accomplished by crushing the tibial nerve. This plan permitted observations on the  $P^{32}$  uptake by totally denervated muscles and by muscles undergoing reinnervation and regeneration.

A condition of prolonged shortening or spasticity in the gastrocnemius muscle of one limb of 49 animals was produced by the injection of suitable doses of tetanus toxin into the popliteal space (3). The doses of tetanus toxin employed had no effect on the contralateral limb. When bilateral spasticity was desired, the same dose of tetanus toxin was injected into each limb. The effects of relaxation upon the phosphate uptake by spastic muscles were investigated by comparing the uptake of  $P^{32}$  by 6-day spastic muscles of 12 unanesthetized animals with the uptake by 6-day spastic muscles of 7 animals in which the spasticity was released while under deep ether anesthesia.

A state of bilateral spasticity was established in 10 animals. One limb was stretched twice daily during which treatments the gastrocnemius was stretched six

times through a full arc of motion. This procedure was begun 2 days after the injection of the tetanus toxin and continued for 12 days prior to the  $P^{32}$  studies. The unstretched spastic muscle of the contralateral limb served as a control.

#### RESULTS

Figure 1 shows the average relative phosphate activity of serum and muscle at 30, 240 and 960 seconds after injection. Inasmuch as it has been shown that  $P^{32}$  enters the intracellular phase of skeletal muscle very slowly (4, 5), it was assumed that the amounts found to be present in muscle represented that in extracellular water. It was assumed that at 960 seconds after injection an equilibrium existed for  $P^{32}$  in serum and muscle. The values for relative and absolute extracellular phosphate space were calculated according to Manery and Bale (5). Our values for extracellular water in the normal gastrocnemius muscle of the rat are in agreement with those obtained by other methods of measurement (5, 6). When the absorption time was 30 seconds the calculated values for extracellular water or phosphate space were only 12.2 per cent of those found when equilibrium was established.

It was shown (fig. 2 and 3) that the right and left gastrocnemii of normal adult rats contained the same amount of  $P^{32}$  at 30 seconds after injection. The muscles which were made to undergo strong vigorous tetanic contractions during the absorption period took up a decreased amount of  $P^{32}$ ; whereas stimulated muscles took up an increased amount of  $P^{32}$  when absorption was allowed to take place immediately after stimulation. The total activity and the activity per gram weight were found to be decreased in the muscles which were subjected to immobilization. Muscles subjected to tenotomy were found to have undergone atrophy but took up the same amount of phosphate as did their controls. Thus, the  $P^{32}$  activity per gram weight of muscle was found to be increased. Studies made on denervated muscles (fig. 5) at 10 days after operation showed a decreased phosphate uptake which paralleled the extent of atrophy. At these times the  $P^{32}$  activity expressed on a gram weight basis was essentially the same as that found in the controls. The experimental muscles at 14, 21, 28 and 42 days after denervation were found to have decreased total phosphate activity but an increased activity per gram weight of muscle.

Muscles made spastic by the injection of tetanus toxin showed less total  $P^{32}$  activity and less activity per gram weight than found in their normal contralateral controls (fig. 4). Relaxation of spastic muscles by means of deep ether anesthesia was followed by a greater uptake of  $P^{32}$  than found in normal controls. Daily treatment of spastic muscles by passive elongation was found to retard atrophy and to increase the phosphate uptake at 14 days following the tetanus toxin injection (figs. 2 and 3).

#### DISCUSSION

In view of the fact that arterial pressure and  $P^{32}$  concentration in the blood is the same for the experimental and control limbs, it is apparent that differences in the amounts of  $P^{32}$  in the control and experimental gastrocnemii must be attributed to factors operating within the muscles. The conceivable variables would include the volume of blood flow, the area and permeability of the capillary bed and, in the cases where atrophy occurred, the volume of extracellular water. It can be estimated from

diffusion calculations that the maximum changes found in our experiments for extracellular fluid space would account for less than 5 per cent of the change in  $P^{32}$  activity. It may be assumed therefore that the relative effective circulatory transport in these experiments primarily depends upon the volume of blood flow and the condition of the capillary bed. It is to be pointed out that although the permeability of the capillary membrane to various diffusible substances is not numerically the same as to  $P^{32}$ ; the circulatory transfer of any diffusible substance, when measured as the percentage of the contralateral control value, would be the same. In this connection

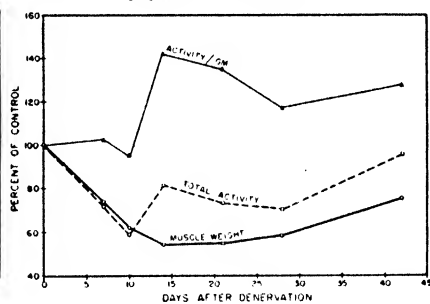
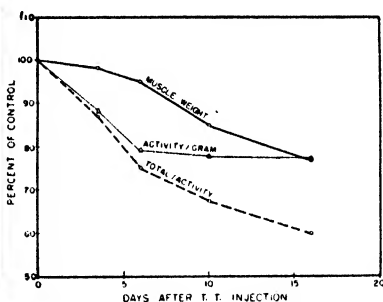
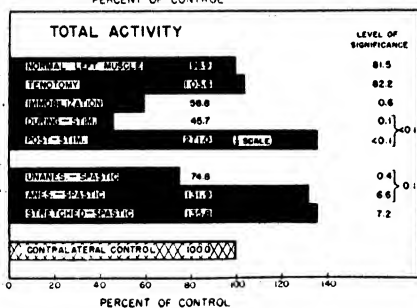
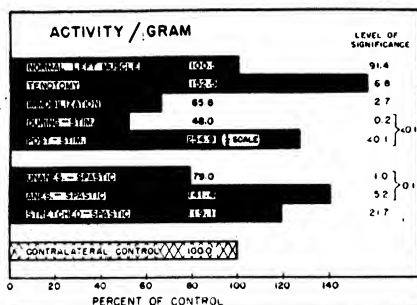


Fig. 2 (upper left).  $P^{32}$  ACTIVITY of experimental gastrocnemii in percentage of that in contralateral control muscles. Relative activity is expressed on gram weight basis.

Fig. 3 (lower left).  $P^{32}$  ACTIVITY expressed on the basis of the amount present in whole muscle.

Fig. 4 (upper right).  $P^{32}$  ACTIVITY of spastic gastrocnemii in percentage of that in contralateral controls at various times after injection of tetanus toxin.

Fig. 5 (lower right).  $P^{32}$  ACTIVITY of experimental gastrocnemii in percentage of that in contralateral controls at various times after crushing or sectioning the tibial nerve.

it is pertinent to point out that the changes in  $P^{32}$  uptake during and following tetanic stimulation are in accord with the predictions that can be made from the results of studies on blood flow in other experiments.

The reduced circulatory transport during a period of tetanic stimulation is attributed to an impediment to blood flow resulting from mechanical compression of the intramuscular blood vessels by the contracted muscle fibers and the increased uptake in the post-stimulation period to reactive hyperemia following partial ischemia. The inactivity associated with immobilization is accompanied by a reduced effective circulatory transport of material to the muscle.

Although the tenotomized muscle is in a shortened state, the lack of active tension may allow a passive dilation of the blood vessels resulting in an increased effective circulation transport. The studies on denervated muscle prior to the onset of reinnervation show a phosphate transfer commensurate with the extent of atrophy. After the onset of reinnervation the phosphate transfer expressed in terms of muscle weight is appreciably increased. These findings suggest that a decreased circulatory transfer is not a critical factor in muscle during either atrophy or regeneration following reinnervation.

Skeletal muscle continuously in a state of reflex hypertonus or spasticity suffers from an impairment in the quantity of diffusible material reaching it by way of the blood stream. This is attributed to a reduction in blood flow resulting from the compression of blood vessels by the shortened muscle fibers. When the spasticity was reduced by deep ether anesthesia, a reactive hyperemia was found to follow the release from the previous state of partial chronic ischemia. The observation that treatment of spastic muscle by passive elongation serves to increase its effective circulation and to retard atrophy suggests that the injurious effects of prolonged shortening on skeletal muscle are in part due to a circulatory deficiency instigated and maintained by the physical state of the muscle.

#### SUMMARY

A study was made of the amount of  $P^{32}$  uptake by gastrocnemius muscles of adult rats under various experimental conditions in which the muscle of one limb served as the experimental and that of the contralateral limb as its control. The muscles were removed for study 30 seconds after an intraperitoneal injection of 0.4 milluries of  $P^{32}$ .

The  $P^{32}$  uptake was the same in the right and left gastrocnemii of normal control animals. Vigorous tetanic contraction of muscle was accompanied by a decreased uptake of  $P^{32}$ ; whereas during the period of relaxation and recovery the uptake was greater than in the controls. Immobilized muscles took up less  $P^{32}$  than their controls. The total phosphate uptake was unaffected by tenotomy but the uptake on a gram weight basis was increased. Denervation was followed by a decreased uptake of  $P^{32}$ , the amounts of which roughly paralleled the degree of atrophy. However, in the longer periods of atrophy the uptake per gram was found to be increased. The condition of prolonged shortening or spasticity in muscle was accompanied by a decreased uptake of  $P^{32}$ . This effect was abolished by deep anesthesia and lessened by daily treatments with stretching. It is postulated that a reduced effective circulatory transfer may contribute to the atrophy and functional impairment found in spastic muscle.

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# FACTORS AFFECTING RATE OF COOLING IN IMMERSION HYPOTHERMIA IN THE DOG<sup>1, 2</sup>

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**P**HYSIOLOGIC observations during reduced body temperature are numerous in the literature with controversial evidence existing on many points. Without doubt a portion of the non-agreement stems from the manner in which the hypothermic state was produced. It seems logical to conclude that, inasmuch as prolonged hypothermia is associated with marked alteration of the functions of many of the systems in non-hibernating, warm-blooded animals, the duration of the condition as well as the rapidity of temperature reduction is probably of prime importance in the recording of any observation.

Methods of lowering body temperature have varied widely. Among these could be mentioned 1) subject placed between rubberized sheets containing coils through which is circulated a cold liquid, 2) exposure to cold air with and without immobilization, 3) ice packs wherein the subject is separated from the ice by a water-proof covering, and 4) immersion of varying portions of the body in water of temperatures ranging from 0° to 20°C. In each of the above methods the type and degree of anesthetization has varied.

Even with a constant method of hypothermia induction in a single species of animal a certain amount of variation exists in the rate of cooling. Access to a large number of temperature records during cooling in the dog made possible an analysis of the relationship of certain factors to the rate of cooling in this species. In addition to shedding some light on certain physiological observations in the hypothermic dog it is believed the findings may likewise have some practical value in the protection of humans accidentally exposed to very cold water.

## METHODS

Unselected mongrel dogs were tied supine to an appropriate cradle after preliminary sedation (usually pentothal sodium). Inasmuch as these data were incidental to other series of observations, the pre-immersion handling varied accordingly. In some cases surgical procedures such as catheterization of various heart chambers or

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<sup>2</sup> These data form a portion of a thesis presented by Mr. Wolff to the Graduate School, Boston University, in partial fulfillment of requirements for the degree of Master of Arts.

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placing of thermocouples in different anatomical sites were accomplished and in others immersion followed immediately on anesthetization. In all cases an endotracheal tube was inserted and a rectal thermocouple inserted to a depth of four inches. Immersion was complete except for the head, neck and ventral portion of the thorax, purposely approximating the exposure of a human floating in a standard life vest. The temperature of the water bath was maintained between 2° and 5°C.

TABLE 1. EFFECT OF SURGICAL PROCEDURES ON COOLING RATE

	NO. OF DOGS	MEAN COOLING RATE °C/MIN. $\pm$ S.D.	MEAN TIME IN MIN. TO COOL 39° C. TO 20° C.
Surgery.....	46	0.140 $\pm$ 0.034	136
Non-surgery.....	31	0.197 $\pm$ 0.080	96

The probability of the above difference in cooling rate being due to chance alone is less than 1 in 1000.

and the temperature at each thermocouple recorded at 24-second intervals by a Leeds & Northrup 'Speedomax' recorder.

In order to standardize the shivering intensity an arbitrary scale was established. The scale, 0 to 4, graded the intensity according to degree of muscular involvement, slight tremors in the head, neck and shoulder regions being graded as 1; regular and rhythmical tremors in the head, neck, forelegs and trunk *grade* 2; general muscular

TABLE 2. EFFECT OF HAIR COAT ON MEAN COOLING RATES IN DOGS

	LONG HAIR	NO.	SHORT HAIR	NO.	DEPILATED	NO.
Surgery.....	0.120 $\pm$ 0.034	23	0.160 $\pm$ 0.030	23	0.172 $\pm$ 0.052	4
Non-surgery.....	0.158 $\pm$ 0.078	11	0.219 $\pm$ 0.074	20	0.212 $\pm$ 0.039	5

Statistical results: Surgery—long hair vs. short hair vs. depilated  $P < 1\%$ . Non-surgery—long hair vs. short hair vs. depilated  $P > 5\%$ . Surgery—long hair vs. short hair  $P < 1\%$ . Non-surgery—long hair vs. short hair  $P < 1\%$ .

twitching through the body *grade* 3; and severe movement of the entire body *grade* 4. In the analysis of the data the time factor was included in the above and each animal classified, in most instances by the same observer.

In several cases repeated immersions were made of the same animal. At least one week separated two exposures of the same dog and rewarming was effected by immersion in warm water (40°–42°C.) for a short interval followed by exposure to room air.

## RESULTS

The immersion period required to reduce the rectal temperature to 20°C. varied from 52 to 227 minutes (average 126). The mean rate of cooling, expressed as °C/min., was computed for each animal. The factors affecting cooling rate which were investigated include pre-immersion surgical procedures, hair covering, shivering intensity, size and sex.

*Pre-Immersion Surgical Procedures.* The considerable effect of surgical procedures in delaying the cooling rate is apparent from table 1. That relatively minor surgery should have such a profound effect on cooling rate is a bit surprising. Hartman (1) has reported a release of epinephrine during and after surgery and since this material is known to increase heat production it is conceivable that the response is at least in part effected in this manner. Too, the adrenal cortex as well as the medulla may play a role in this phenomenon in line with the stress response of this structure.

An increase in depth of anesthesia required for surgery might be suspected of affecting the result. But, as reported previously (2), an increase in anesthetic depth tends to facilitate cooling rather than reduce it as here found in the surgically treated animals.

TABLE 3. SUMMARY OF ANALYZED DATA FOR GRADED SHIVERING INTENSITY

SUB-GROUP	GRADED SHIVERING INTENSITY				
	0	1	2	3	4
Non-surgery, long hair		0.217 ±0.01072 (4)	0.110 ±0.0212 (2)	0.125 ±0.0475 (2)	0.133 ±0.0145 (3)
Non-surgery, short hair	0.203 ±0.0336 (4)	0.281 ±0.0719 (7)	0.168 ±0.0532 (4)	0.207 ±0.0730 (3)	0.154 ±0.0574 (2)
Surgery, long hair	0.144 ±0.0306 (3)	0.109 ±0.0150 (5)	0.125 ±0.0244 (5)	0.117 ±0.0207 (8)	0.111 ±0.0170 (2)
Surgery, short hair	0.173 ±0.0264 (6)	0.164 ±0.0360 (5)	0.162 ±0.0219 (6)	0.148 ±0.0328 (5)	0.106 ±0.0000 (1)
Combined results	0.175 ±0.0368 (13)	0.200 ±0.0915 (21)	0.146 ±0.0369 (17)	0.146 ±0.0478 (18)	0.129 ±0.0314 (8)

Mean rates of cooling in °C/min.; number of dogs in parentheses.

*Hair Covering.* Spealman (3) reported no effect of depilation on cooling rate of an unanesthetized dog immersed in water of 0°C. Also Haterius and Maison (4) found no appreciable effect in a few early trials in this laboratory. But when a large number of dogs were grouped according to long hair, short hair and depilated a significant difference was noted. These results are shown in table 2. Since surgical procedures have been shown to affect cooling rate the data on hair coat are separated into surgical and non-surgical groups. The numbers in the corresponding groups are not proportionate, making it seem inadvisable to pool the data of the surgical and non-surgical groups. An independent analysis of variance of the surgical and non-surgical groups reveals high significance ( $P < 1\%$ ) in the former but non-significance in the latter. Inspection of the table shows that the small reversal of order between short-hair and depilated dogs doubtless causes this result. If, on the other hand, long-haired dogs are compared with either short-haired or depilated, the difference is significant ( $P < 5\%$ ) in both groups. This result is easily explained on the basis that long hair

tends to mat down when wet, forming a protective layer about the dog while short hair offers little or no more protection from the cold water than no hair.

*Shivering.* The effect of shivering intensity on cooling rate is apparent from table 3. The data are presented according to subgroups of previously discussed factors, i.e. surgery and hair coat. But for analysis the data are pooled since inspection reveals a relatively proportional contribution of each of the subgroups to the total. An analysis of variance of the combined totals reveals a highly significant difference ( $P < 1\%$ ) among the cooling rates.

Of especial interest in this table is the faster cooling rate of those dogs showing *grade 1* shivering as compared with the non-shivering group. This difference, although not quite statistically significant, lends itself to interesting speculation. Two possible explanations present themselves if the phenomenon be real. These are 1) the shivering motions of the dog create convection currents in the water bath thereby making the contact layer cooler and 2) the minimal shivering motions facilitate peripheral circulation in the dog thus causing a better conduction of the heat to the surface, whereas heat production is increased only slightly.

TABLE 4. EFFECT OF BODY SIZE ON COOLING RATE OF DOGS

WEIGHT	NO.	COOLING RATE °C/MIN.	TIME IN MIN. TO COOL FROM 39° TO 20° C.
0-9.0	24	$0.175 \pm 0.0357$	100
10-14.9	29	$0.163 \pm 0.0414$	117
15 up	24	$0.151 \pm 0.0433$	126

*Body Size.* The effect of body size on cooling rate is shown in table 4. Although a definite trend exists toward slower cooling with the larger dogs the results are not statistically significant. It is possible that the effect of weight was masked in this analysis by the surgical, hair coat and shivering factors, but when broken down into all these sub-groups the number of animals in any block becomes too few for valid conclusions.

*Sex.* Comparison of male with female cooling rates, either in sub-groups or as totals, failed to reveal any appreciable difference.

#### DISCUSSION

A considerable difference in rate of body heat loss, even among animals of the same species when subjected to the same cold environment, is a common observation among workers in the field of hypothermia. The ultimate prognosis of two animals with appreciable different cooling rates may likewise be quite different or may, except for time, be similar. In the interest of more careful control of the conditions a knowledge of the various factors concerned with cooling of the animal would appear to be necessary.

In a previous paper (2) the effect of variations in metabolic rate on the cooling rate at any instant as well as the overall effects of anesthetic depth were discussed. In the present paper several other factors have been shown to play a role in the rate of temperature loss in the immersed dog. It would appear that in any work in



which the time of induction of the hypothermic state is a factor the conditions of size, hair coat, depth of anesthesia, surgical maneuvers, and shivering intensity and time must be carefully controlled.

#### SUMMARY

A statistical study has been made on the factors which affect the rate of cooling of anesthetized dogs immersed to the neck in an ice bath. The factors investigated include surgical manipulations, hair covering, shivering intensity, body size and sex. Even relatively slight surgical maneuvers were found to significantly decrease the rate of cooling. It is suggested that the adrenal gland may contribute to this response. A heavy coat of hair offered significant protection against cooling but short hair proved no better than no hair. Shivering was found to have a significant effect in slowing the cooling rate, but a minimal shivering showed a tendency toward an increased cooling rate when compared with no shivering. Although a definite trend toward slower cooling with increasing body size exists the differences were not found to be statistically significant. No difference between sexes was detected in the cooling rate.

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# THERMAL REGULATION DURING ACCLIMATIZATION IN A HOT, DRY (DESERT TYPE) ENVIRONMENT

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NIELSEN (1) showed that when work is performed at a constant metabolic rate the rectal temperature rises to a plateau value, which is determined by the metabolic rate and is independent of the environmental conditions, unless these are extreme. This suggested that the rectal temperature is under physiological control and that its elevation during work is not the result of inability to dissipate the increased metabolic heat. If such an inability did exist, the rectal temperature would be higher in hot environments, where heat loss is retarded, than in cool environments where heat loss is more rapid. Although differences in rectal temperature did not occur during similar work rates in the usual ranges of environmental temperatures, Nielsen (1) observed that in very hot environments normal control of the rectal temperature could not be maintained.

When man works for the first time in a hot environment his body temperature rises to abnormally high levels and there occur associated psychomotor, gastro-intestinal and particularly circulatory disturbances which often prevent effective work and at times produce outright disability (2-6). On repeating the work in the heat, he becomes acclimatized and develops adaptive mechanisms which permit him to perform a given work at nearly the same rectal temperature in the heat as in the cool (2-6). The process of acclimatization restores the normal relationship whereby once again the rectal temperature is determined by the metabolic rate. Concomitantly, the undesirable disturbances of the unacclimatized state are reduced, or fail to appear, and subjectively the man works nearly as easily in the hot environment as in the cool one.

This paper deals with a study of the thermal balance during acclimatization in a hot, dry environment.

## METHODS

Three aspects of thermal balance were studied:

1) *Heat content and heat distribution of the body.* Changes in rectal temperature were used as indices of changes in the average temperature of the deep tissues (7)

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and variations in mean skin temperature as indices of changes in temperature of the peripheral tissues. Changes in the average temperature of the body as a whole, and in its heat content, were estimated by the formulae of Burton (7) and of Hardy and DuBois (8) which combine changes in both rectal and mean skin temperatures.

2) *Heat interchanges between man and his environment.* Partitional calorimetry (9-11) was used to determine the total thermal balance and the thermal interchanges

TABLE I. HEART RATE, RECTAL TEMPERATURE AND MEAN SKIN TEMPERATURE AT END OF ONE HOUR, AND AVERAGE SWEAT RATE AND 'PERIPHERAL BLOOD FLOW' FOR ONE HOUR, OF WORK IN A COOL AND IN A HOT, DRY ENVIRONMENT (DATA ARE AVERAGES FOR THREE MEN)

	DAY	HEART RATE/MIN.	RECTAL TEMP. (T <sub>r</sub> )°C.		MEAN SKIN TEMP. (T <sub>s</sub> )°C.		SWEAT RATE	'PERIPHERAL BLOOD FLOW' AVERAGE OVER HR.
			Final	Change	Final	Change		
Initial cool control	5	102	37.9	0.7	30.8	-2.5	76	.34
	8	112	38.0	0.7	31.1	-2.0	86	.38
	9	111	37.8	0.8	30.9	-2.7	79	.35
Hot environment	1	162	39.0	1.6	37.8	3.3	621	2.58
	2	157	38.5	1.3	37.5	3.1	639	2.63
	3	143	38.4	1.0	37.1	2.4	666	2.28
	4	131	38.3	1.0	37.0	2.5	669	2.04
	5	129	38.2	1.1	36.7	2.3	665	2.23
	6	121	38.1	0.9	36.4	2.1	678	1.90
	8	127	38.0	0.7	36.4	2.3	682	1.82
	9	123	37.9	0.5	36.2	1.6	683	1.54
	10	118	37.9	0.5	36.4	1.7	692	1.76
Final cool control	1	103	37.7	0.4	31.1	-2.7	83	.37
	2	100	37.7	0.5	31.1	-2.5	71	.36

by each of the avenues of thermal exchange. These interchanges have been expressed in the heat-flow equation (9-11):

$$^4 \Delta H/t = M - E \pm C \pm R$$

where  $\Delta H/t$  = rate of change of heat content of the body when  $H$  = heat content and  $t$  = time

$M$  = rate of metabolic heat production

$E$  = rate of evaporative heat loss

$C$  = rate of heat exchange with surrounding air by convection

$R$  = rate of heat exchange with the environment by radiation

positive when skin temperature is below environmental temperature, negative when above.

Heat interchanges by conduction are usually small and are omitted from consideration.

<sup>4</sup> The 'storage' of many authors.

3) *Flow of heat within the body.* Heat flows to the periphery of the body by conduction through tissues and by transport by the blood stream. Changes in 'peripheral blood flow' were estimated by the formula of Hardy and Soderstrom (12) which gives the flow to surface tissues to an average depth of 2 centimeters.

*General Plan of the Experiment.* The temperatures and thermal interchanges of 3 healthy young men were determined during one hour of work on successive days as they became acclimatized in a hot, dry environment. First, the men were physically conditioned by road marches and treadmill walking during a period of 14 days. Then followed 9 days of test activity in a cool environment. The 5th, 8th and 9th days became the control, cool days. The next 10 days were 'hot days' and on 9<sup>th</sup> of these test activity was carried out. On the 2 days following heat exposure, the activity was repeated in the cool environment.

*Test Activity.* Immediately after the base-line rectal temperature, skin temperatures, pulse rate and blood pressure were determined in a control room (D.B.<sup>6</sup> 78°F.) the men entered the test environment and underwent *the test hour* of work. This was always their first work and environmental exposure of the day and was carried out at a fixed hour each day for each man. After the test hour they rested for 4 hours and then, in order to maintain physical fitness and acclimatization, they marched with 20-pound packs for the last 2 hours of exposure. Following these 7 hours of regulated activity in the test environment the men returned to their temperature-controlled 'quarters,' (D.B. 78°F.).

*Test Hour.* The activity was stop-watch timed and consisted of five 10-minute periods of walking on a treadmill in a wind tunnel, with each period followed by a 2-minute interval during which the men stepped off the mill and were weighed on a balance. During the weighing, salted water (0.1% NaCl) at body temperature was drunk in amounts equal to the sweat lost. The rate of walking was 2.5 miles per hour, up a 2.5 per cent grade and facing the wind stream. The men were nude except for shoes and socks. During the hour the following measurements were made: air temperatures and wind velocity during the first 3 minutes of each period; skin temperatures and heart rate during the last 4 minutes of each period; metabolic rate during the last 8 minutes of the first, second and fifth periods; weight, rectal temperature and water intake during each weighing interval.

*Test Environments.* The characteristics of the control, cool environment were D.B. 78°F., W.B.<sup>7</sup> 62°F., R.H.<sup>7</sup> 39 per cent; of the hot, dry environment D.B. 123°F., W.B. 80°F., R.H. 15 per cent. In both environments, the radiation temperature of the blackened tunnel walls equalled the air temperature and the air movement was 450 feet per minute in a linear, horizontal flow.

*Methods of Measurement: Environment.* Dry-bulb temperature ( $T_a$ ) and wet-bulb temperature of the air were measured by calibrated mercury thermometers; air movement by a hot wire anemometer and an Alnor velometer; radiation temperature by a radiometer successively directed at the 6 tunnel surfaces, with the average of the 6 readings taken as the radiant temperature ( $T_{re}$ ).

<sup>5</sup> A curtailed program was substituted on the 7th hot day, a Sunday.

<sup>6</sup> D.B. = dry-bulb temperature.

<sup>7</sup> W.B. = wet-bulb temperature, R.H. = relative humidity.

**Body temperatures.** Rectal temperature ( $T_r$ ) was measured by calibrated clinical thermometers. The skin temperatures of 6 areas were determined by a radiometer (13) and each reading was weighted according to the area of skin represented (14, 15). The weighting factors of omitted skin areas were assigned to measured areas found by preliminary study to have similar temperatures. The final weighting factors were;

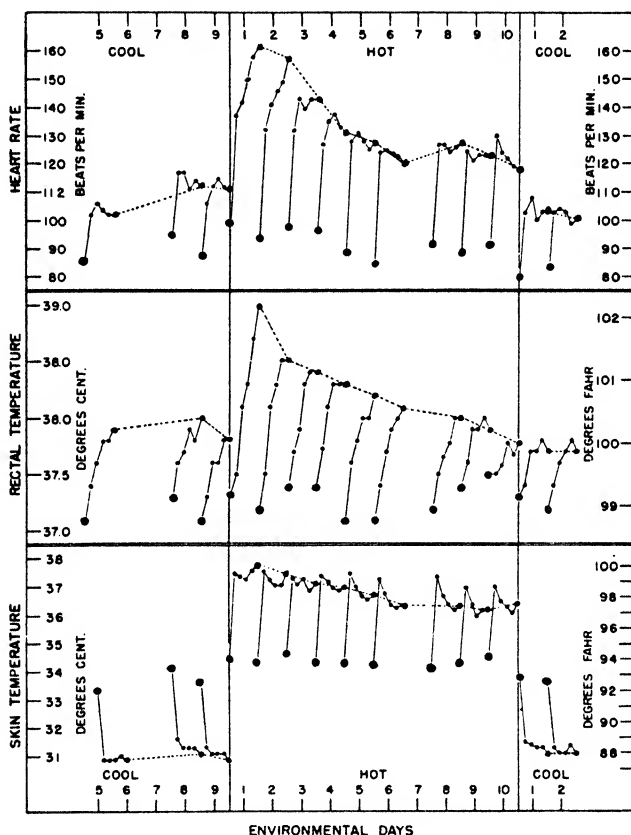


Fig. 1. HEART RATE, rectal temperature and mean skin temperature during one hour of work in a cool environment and during acclimatization in a hot, dry environment. Solid lines connect base line determinations taken at rest in a cool environment (large points) with 5 successive determinations (small points) taken during test hour of work on each day; broken lines connect final readings from day to day.

cheek 0.14, forearm 0.14, palm 0.05, thigh 0.32, back 0.17, chest 0.18. The sum of the final weighted readings gave the mean skin temperature ( $T_s$ ).

**Oxygen consumption.** Exhaled air was collected in a gasometer and duplicate aliquots were analyzed for  $O_2$  and  $CO_2$ .

**Weight changes.** A beam balance sensitive to 4 grams was used.

**Heart rate.** The radial pulse was counted.

## CALCULATIONS

Calculations were made from the data of each 12-minute period (10 minutes of work plus the 2 minutes of the succeeding weighing) with the assumption that these data applied to the overall activity of the entire period. All calculations were made for each subject, corrected for surface area, and combined into an average value for the three men.

*Changes in Body Heat Content.* The formula of Burton (7) was used. The specific heat of the body was taken as 0.83 and the partition between the deep and peripheral tissues as 0.67 and 0.33, respectively.

$$\Delta H_{\text{total}} \text{ in Cal.} = (0.67 \Delta T_r^{\circ}\text{C.} \times \text{wt. in kg.} \times 0.83) \\ + (0.33 \Delta T_s^{\circ}\text{C.} \times \text{wt. in kg.} \times 0.83)$$

where the first parenthesis gives the change in heat content of deep tissues and the second parenthesis the change in heat content of peripheral tissues.

*Heat Flows. Metabolic heat production (M)* was calculated from the oxygen consumption using the caloric equivalent of oxygen at the measured R.Q. and correcting for the external work performed. Since the metabolism for the 3 measured periods varied by only 3 per cent, the average of these periods was taken as the heat production for each of the 5 periods.

*Evaporative cooling (E)* was calculated from the total water loss (skin and lungs) and the latent heat of vaporization of water (0.576 Cal/gm.) at skin temperature. Water loss was obtained from the change in weight, corrected for the water ingested and for the excess weight of the CO<sub>2</sub> exhaled over the O<sub>2</sub> consumed. This calculation is permissible since no sweat was lost by dripping.

*Convection (C) and Radiation (R)* were calculated as the sum of both factors ( $C + R$ ). First an empirical coefficient of convection plus radiation exchange,  $K_{C+R}$ , was determined for each of the two environments studied. According to the heat-flow equation,  $M - E \pm C \pm R = 0$  or  $C + R = -(M - E)$  when there is no change in body heat content. By solving the equation for  $C + R$  for those periods in which there was no change in either rectal or mean skin temperature, then dividing each  $C + R$  by the difference between the air and skin temperature at that time, a series of coefficients was obtained.

$$K_{(C+R)} \text{ in Cal/m}^2\text{/hour/}^{\circ}\text{C.} = \frac{-(M - E) \text{ Cal/hour}}{(T_a^{\circ}\text{C.} - T_s^{\circ}\text{C.})(\text{m.}^2)}$$

In the hot environment the average of the coefficients  $K_{C+R}$  thus calculated was 18 Cal/m<sup>2</sup>/hour/°C. and for the cool environment, 25 Cal/m<sup>2</sup>/hour/°C.

These coefficients were then used to calculate the  $C + R$  interchanges for all periods according to the differences between air and mean skin temperature, regardless of changes in body heat content. The constancy of the conditions and subjects of the experiment permits the derivation and use of these coefficients in this study.

A rough partition of  $C$  and of  $R$  was attempted by making an independent calculation of  $R$  (14). The radiation area of walking man was assumed to be 0.9 of the surface area and the emissivities of the skin and of the tunnel walls were taken as 1.

Heat interchange by conduction was not estimated and it is therefore included in the  $C + R$  calculations above.

*Net Rate of Thermal Flow.* The individual channels of thermal flow were then summated algebraically, according to the heat-flow equation, to give the net rate of heat gain or heat loss to the body,  $\Delta H/t$ . When the *average net rate per hour* is thus calculated one obtains a value for thermal balance which can be compared with the change in body heat content at the end of one hour, as calculated from changes in rectal and mean skin temperatures. Two evaluations of thermal balance are thus obtained.

'*Peripheral Blood Flow*'. The average blood flow over the test hour was calculated (12). Although these values may not be quantitatively accurate, they serve as useful indicators of changes in blood flow in the surface tissues.

$$PF = 17 \left( \frac{M - \Delta H/t}{T_r - T_s} - K_{cd} \right)$$

$PF$  = blood flow, ml/m<sup>2</sup>/minute.

$K_{cd}$  = thermal conductivity of peripheral tissues, Cal/m<sup>2</sup>/hour/°C., 9.1 for males.

17 = factor converting Cal/m<sup>2</sup>/hour/°C. to ml/m<sup>2</sup>/minute.

## RESULTS

The results are presented in terms of the average values for the three men<sup>a</sup>.

*General Reactions.* The characteristic reactions of unacclimatization and acclimatization were observed in all subjects (2-6). On *hot day 1* the men completed the test hour with great difficulty and 2 men approached complete exhaustion. On the second day they experienced slightly less difficulty and thereafter improved steadily. On *day 10* the men performed the work easily and their appearance suggested no strain greater than that incurred in the control cool environment.

*Heart Rate (Table 1, Fig. 1).* In the cool environment the heart rate increased moderately as work was begun and then remained at that level for the rest of the test hour. On *hot day 1*, in contrast, the heart rate increased with each successive work period and reached 162 per minute at the end of the hour. As acclimatization developed the final heart rate progressively decreased, almost entirely as a result of plateauing at the level reached at the end of the first work period. In this respect the heart rate returned to the pattern of its response in the cool environment. On *hot day 10* the final heart rate was 118 per minute, 15 beats per minute higher than the rate in the cool, an indication that the hot environment was still imposing a greater stress than the cool one on the circulatory system.

*Rectal Temperature (Table 1, Fig. 1).* In the cool environment the rectal temperature rose slightly during the first work periods, then plateaued in the later periods at a level of 37.8°C., the normal deep tissue temperature for the work performed. In contrast, on *hot day 1* the rectal temperature rose progressively with each successive

<sup>a</sup> Three tables of detailed data for this paper have been deposited with the American Documentation Institute, 1719 N Street, N.W., Washington 6, D. C. For copies of these tables order Document 2935 directly from American Documentation Institute, remitting \$0.50 for microfilm (images 1 inch high on 35-mm. film) or \$0.50 for photocopies (6 x 8 inches).

work period and reached 39.0°C. at the end of the hour, 1.2°C. above that attained in the cool environment. On the second hot day the temperature rose less and leveled off in the last period. On the subsequent days, the rise in temperature decreased progressively and the leveling in the latter periods became more pronounced, so that on *hot day 10*, the final rectal temperature was 37.9°C., only 0.1°C. above the final value in the cool.

*Mean Skin Temperature* (Table 1, Fig. 1). In the cool environment, the mean skin temperature fell sharply during the first work period, then leveled off at this value to give a final temperature of 31.0°C. The difference between the average rectal and

TABLE 2. INDIVIDUAL RATES OF THERMAL FLOW AND THERMAL BALANCE DURING ONE HOUR OF WORK IN A COOL AND IN A HOT, DRY ENVIRONMENT (DATA ARE THE AVERAGES FOR THREE MEN)

	DAY	M	E	C + R	R	NET RATE OF <sup>1</sup> THERMAL CHANGE	CHANGE BODY HEAT <sup>2</sup> CONTENT/ HR.
		Cal/m <sup>2</sup> /hr.	Cal/m <sup>2</sup> /hr.	Cal/m <sup>2</sup> /hr.	Cal/m <sup>2</sup> /hr.	Cal/m <sup>2</sup> /hr.	Cal/m <sup>2</sup>
Initial cool control	5	188	-44	-134	-25	2	-10
	8	187	-50	-143	-27	-6	-16
	9	182	-45	-140	-27	-3	-12
Hot environment	1	180	-358	235	76	57	67
	2	177	-369	240	77	48	57
	3	177	-384	243	78	37	44
	4	178	-384	240	78	34	45
	5	177	-382	246	79	41	46
	6	173	-390	250	80	32	40
	8	175	-392	252	81	35	38
	9	172	-393	251	81	30	27
	10	173	-398	254	82	28	27
Final cool control	1	178	-48	-142	-27	-12	-20
	2	179	-41	-140	-27	-2	-15

All heat gains to the body are positive, all heat losses negative (-). <sup>1</sup> Calculated from heat-flow equation. <sup>2</sup> Calculated from changes in mean skin and rectal temperatures.

average mean skin temperatures for the test hour was about 6.6°C. and this constituted the internal thermal gradient for the flow of the heat from the deep to the surface tissues.

On *day 1* in the heat the mean skin temperature rose sharply during the first work period to 37.5°C. and during the remainder of the hour rose slightly higher, to 37.8°C. On the following hot days the initial steep rise became only slightly smaller but in the later periods the temperature tended to fall from the high value of the first period and after *day 5* this fall became pronounced. As a result, on *hot day 10* the final mean skin temperature was 36.4°C., 1.4°C. below that on *day 1*. Moreover, since the fall in skin temperature over the 10 hot days was greater than the parallel fall in rectal temperature, the difference between the average rectal and average mean skin temperatures for the hour increased from 0.8° to 1.3°C., and to this extent



the internal thermal gradient was increased. Nevertheless, the gradient was still considerably less than that ( $6.6^{\circ}\text{C}.$ ) in the cool environment.

'Peripheral Blood Flow' (Table 1). In the cool environment the average 'peripheral blood flow' over the test hour was approximately  $0.3 \text{ l/m}^2/\text{minute}$ . In the heat, the values for the individual men varied considerably but on the average an eight-fold increase to approximately  $2.6 \text{ l/m}^2/\text{minute}$ , occurred on *day 1*. Thereafter the blood flow fell progressively to approximately  $1.5 \text{ l/m}^2/\text{minute}$  on *hot day 10*, still a five-fold increase above that in the cool.

Heat Content (Table 2). In the cool environment the total body heat content decreased by  $13 \text{ Cal/m}^2$  during the test hour, due to the large fall in peripheral (skin) temperature which outweighed the accompanying rise in deep (rectal) temperature. In contrast, on *hot day 1* the total body heat content increased by  $67 \text{ Cal/m}^2$ . This increase became progressively smaller with acclimatization, but still amounted to 27

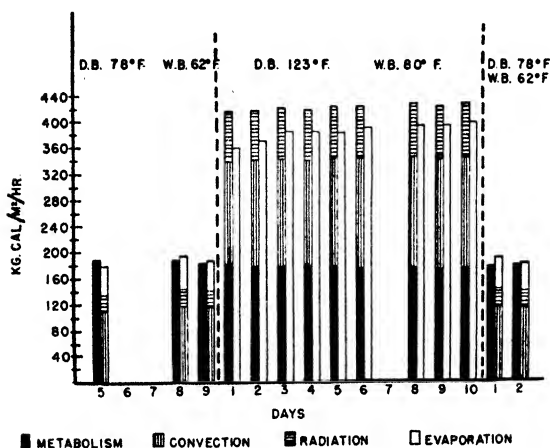


Fig. 2. COMPARISON of thermal balance during work in cool and in hot, dry environment. Each pair of columns represents thermal flow on one day; left column of each pair indicates channel and magnitude of each of thermal inflows to body, right column of each pair shows in a similar fashion the thermal outflows.

$\text{Cal/m}^2$  on *day 10*,  $40 \text{ Cal/m}^2$  above the final heat content in the cool. On all hot days rises in both the deep and peripheral temperatures contributed to this increase.

When the changes in heat content were partitioned between the deep and peripheral tissues, then, in the cool environment the deep tissue heat content increased by  $14 \text{ Cal/m}^2$  during the test hour. In the heat, the deep tissue heat content increased by  $33 \text{ Cal/m}^2$  on *day 1* but on *day 10* the increase was only  $11 \text{ Cal/m}^2$ , essentially the same as in the cool environment. Accordingly, the difference of  $40 \text{ Cal/m}^2$  between the total body heat content on completing work, in the cool and when acclimatized in the heat, was due to the difference in heat content of the peripheral tissues in the two environments.

Thermal Balance (Table 2, Fig. 2). In the cool environment, where metabolic heat production was the sole channel of heat gain and convection, radiation and evaporation were avenues of heat loss, a slightly negative thermal balance resulted. In the hot environment, metabolic heat production remained essentially unchanged but convection and radiation now became channels of thermal inflow of great magni-

tude. Evaporative cooling was the sole avenue of heat loss and although its rate increased eight-fold, the heat gains were not balanced and a positive thermal inflow resulted. The inflow amounted to 57 Cal/m<sup>2</sup>/hour on *day 1* and decreased to 28 Cal/m<sup>2</sup>/hour on *day 10*. These rates of heat inflow are in reasonable agreement with the values for changes in body heat content (67 Cal/m<sup>2</sup> on *day 1* and 27 Cal/m<sup>2</sup> on *day 10*) calculated from changes in rectal and mean skin temperatures. Thus a check was afforded of the changing thermal balances during acclimatization.

*Individual Thermal Interchanges* (Table 2, Fig. 3). The rate of metabolic heat production decreased slowly during acclimatization and on *hot day 10* was 7 Cal/m<sup>2</sup>/hour less than on *day 1*. However, on *day 4* when subjective improvement was already marked and when the increase in heat content over the test hour was 23 Cal/m<sup>2</sup> less than on *day 1*, the metabolic heat gain had decreased by only 2 Cal/m<sup>2</sup>/hour—hardly a significant contribution to the improved thermal balance. The rate of heat gain by convection and radiation actually increased during acclimatization and on *hot day 10* amounted to 19 Cal/m<sup>2</sup>/hour more than on *day 1*. Two-thirds of this increase could be attributed to convective gain, one-third to radiation gain. Summating the changes in metabolism, convection and radiation we find that on *hot day 10* the total heat gain attributable to them was 12 Cal/m<sup>2</sup>/hour greater than on first exposure to heat.

Increased evaporative cooling restored thermal balance as acclimatization developed. Over the 10 hot days, evaporative cooling increased by 40 Cal/m<sup>2</sup>/hour and its most rapid increase, 26 Cal/m<sup>2</sup>/hour, occurred in the first 4 days when acclimatization was also developing most rapidly. Moreover, both in magnitude and in time relationships, the curve of decreasing net rate of heat gain coincided well with the curve of increasing evaporative cooling (fig. 3).

A comparison of the individual rates of heat interchange by periods within the test hour indicates how thermal balance was restored as acclimatization developed (fig. 4). The net result of these flows was that on *day 1* heat was gained during each period; at a high rate during *period 1*, at a somewhat reduced, but considerable, rate (27–40 Cal/m<sup>2</sup>/hour) during the remaining 4 periods. Heat flowed into the body throughout the hour and the body temperatures rose steadily (fig. 1). In contrast, on *day 10* heat was gained during the first period at almost as high a rate as on *day 1* but thereafter the net rate of thermal flow fell practically to zero as the rate of evaporative cooling increased in the last 4 periods. Only in the first 12 minutes was there a positive thermal imbalance and it was during this period only that the body temperatures rose. In the remainder of the hour the increased evaporative cooling restored thermal equilibrium and the body temperatures remained stabilized (fig. 1).

#### DISCUSSION

One may postulate the following events when an unacclimatized man works in a hot environment. The body gains heat rapidly, at the surface by convection and radiation and internally by metabolic heat production. Due to the high environmental temperature the skin temperature rises. This results in a reduction in the external thermal gradient and hence in a lessening of the environmental heat gains. On the other hand, the internal thermal gradient is also decreased. Heat flow from deep to

surface tissues is impaired and deep tissue temperature rises. Sweating becomes profuse but the increased evaporative cooling proves inadequate to dissipate all of the heat gains and deep tissue temperature rises progressively. Because of the small internal thermal gradient a large 'peripheral blood flow' is required to transport to the surface the heat which is dissipated, leaving an inadequate blood flow for the needs of all of the other organs. The two cardinal manifestations of the unacclimatized state have resulted, 1) an elevated body temperature and 2) an overtaxed, unstable circulation.

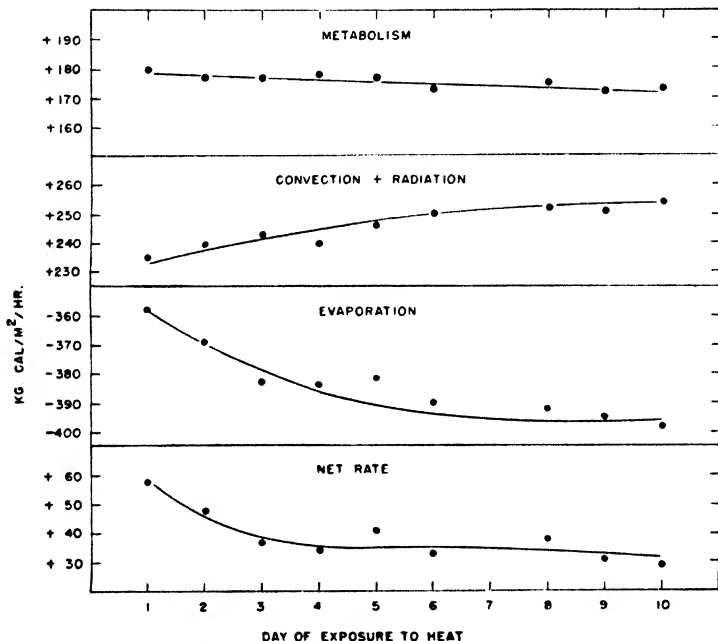


Fig. 3. ANALYSIS of changes in individual channels of thermal flow during development of acclimatization in a hot, dry environment. Average value for test hour of work is plotted for each of the 10 days in hot, dry environment.

During acclimatization thermo-regulating adjustments develop so that body temperature is again regulated with respect to metabolic rate (1) and rectal temperature returns to the level attained when the same work was performed in a cool environment. Moreover, only deep tissue temperature is so regulated. Thus, equal increases in deep tissue heat content occurred in the cool environment and when acclimatized in the heat, in contrast to the markedly different heat contents of the peripheral tissues in the two environments. This substantiates Nielsen's (1) findings that the temperature of the deep tissues, or some part of these tissues, is normally regulated by the metabolic rate of a given task and is apparently critical for normal physiologic function. Skin temperature is not similarly adjusted, may vary widely, and tends to approach environmental temperature (1, 16). The tolerance of the skin for large tem-

perature changes serves temperature regulation in two ways; *a*) changes in peripheral heat content, occurring without significant changes in deep tissue temperature, temporarily buffer critical deep tissue temperature against sudden, transient changes in environmental heat loads, *b*) the change in skin temperature toward environmental temperature diminishes heat interchanges with the environment.

The principal adaptation of acclimatization which returned deep tissue temperature to normal was increased sweat secretion. The resultant increase in evaporative cooling largely restored the thermal balance, accounting for 90 per cent of the reduction in body heat content achieved by *day 4* and for at least 75 per cent of the reduction on *day 10*. The small decrease in metabolic heat production (4%) played at best a minor role but could have accounted for approximately one-fourth of the fall in body heat content achieved by *day 10*. Moreover, its slow, steady fall differed from the initially more rapid changes observed in all other indices of acclimatization.

A consequence of the acclimatization process was that the major factor which restored thermal balance concomitantly increased the environmental heat gains. The increased evaporative cooling lowered the skin temperature, widened thereby the external thermal gradient and as a result heat gains by convection and radiation increased. On *hot day 10* these gains were 8 per cent greater than on *day 1* and to this extent the same environment became physiologically more severe as acclimatization developed. Because of the increased environmental heat gains twice as large an increase in sweat secretion from *day 1* to *day 10* was required to restore thermal balance as would have been required if the greater evaporative cooling had not increased the environmental heat gains. Even so, thermal balance was restored by an increase of only 10 per cent (about 125 ml/hour) in the total sweat rate of 1200 to 1300 ml/hour encountered in this environment.

The increased environmental heat gains in the acclimatized state are properly considered in conjunction with the improved circulation. Both resulted from the lowering of the skin temperature by the increased evaporative cooling. The proportionately greater fall in skin temperature than in rectal temperature increased the internal thermal gradient and accelerated the flow of heat to the surface. The specific thermal conductivity of tissues<sup>9</sup> is so poor that the increase in the internal thermal gradient from 0.8°C. on *hot day 1* to 1.3°C. on *day 10* probably did not increase significantly heat transfer by conduction through the tissues. However, this small increase in the internal gradient made heat transfer by the blood stream considerably more effective, permitting 0.62 liter of blood to transfer the heat previously carried by 1.0 liter of blood. The actual reduction in flow was somewhat less, about 30 per cent. The reduced blood flow, nevertheless, adequately carried to the surface the excess deep heat, and rectal temperature did not rise. The 'spared' blood was made available to the rest of the circulation which now proved adequate for all needs and a stable total circulation resulted.

We may now summarize what the process of acclimatization achieves. Deep tissue temperature is returned to the normal level set by the metabolic rate of the task in a cool environment, but neither total body temperature nor mean skin tem-

<sup>9</sup>  $K = 0.0004 \text{ cal/cm}^2/\text{second}/^\circ\text{C.}$ , approximately that of wood (17).

perature are returned to their levels in the cool environment. Mean skin temperature is adjusted to a level which permits thermal equilibrium between the body and the environment on the one hand, and on the other, maintains an internal thermal gradient which permits the transport of the deep heat to the surface without overtaxing the circulation. In the hot, dry environment of this study these conditions were attained almost wholly as a result of the increased evaporative cooling which an increased sweat secretion produced.

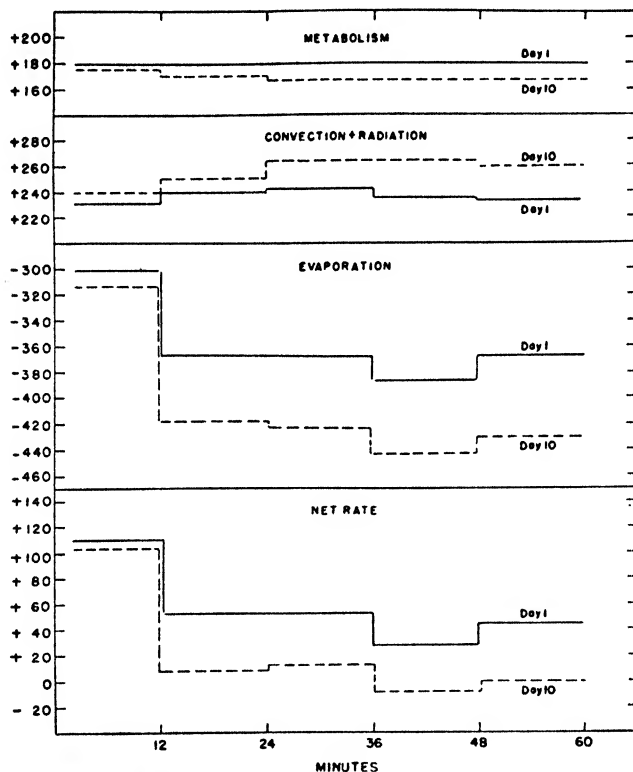


Fig. 4. THERMAL BALANCE in acclimatized state as indicated by comparison of individual avenues of thermal flow during test hour before and after acclimatization. Rates of gain and loss of heat to the body are shown by 12-minute periods during test hour for first and tenth days of work in a hot, dry environment.

#### SUMMARY

The acclimatization process returned the elevated deep tissue (rectal) temperature to the normal level determined by the metabolic rate of the task in a cool environment and set the peripheral tissue (mean skin) temperature at a level which permitted thermal equilibrium between the body and environment on the one hand, and on the other, permitted the transport of deep heat to the periphery without overtaxing the circulation.

The principal adaptive mechanism of acclimatization which produced these changes was a *further increase* (10%) in sweat production. The resultant increase in evaporative cooling accounted for 75 to 90 per cent of the reduction in body temperature and heat content. A small decrease (4%) in metabolic heat production accounted for only 5 to 20 per cent of the reduction in body heat content.

The increased evaporative cooling of the acclimatized state lowered the skin temperature and produced thereby two dissimilar effects: *a*) heat gains by convection and radiation increased (8%) and this necessitated twice as large an increase in sweat secretion as would have been required had the skin temperature not fallen, *b*) the internal thermal gradient widened so that a smaller (70%) 'peripheral blood flow' sufficed to transfer the required deep heat to the periphery.

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# CHANGES IN POTASSIUM AND SODIUM CONCENTRATIONS IN LIVER SLICES ACCOMPANYING INCUBATION IN VITRO

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IT HAS been demonstrated that liver slices when incubated in a potassium-rich medium containing glucose or pyruvate synthesize glycogen much more readily than slices incubated in a sodium-rich medium (1-3). From this, it has been inferred that a potassium-rich medium preserves the intracellular concentration of potassium more effectively than does a low potassium, high sodium medium, such as bicarbonate-Ringer solution.

However, no data were presented in the experiments referred to above on what changes actually occurred in the tissue sodium and potassium during the incubation of the liver slices *in vitro*. Because of the importance of such information in interpreting the results on carbohydrate metabolism by the liver, experiments have been undertaken to provide such data.

Several observations on the effect of varying the ionic environment on the potassium of muscle have been reported previously. Boyle and Conway (4) found that it was necessary to raise the potassium chloride concentration (substituted progressively for sodium chloride) to 29 mM per liter to prevent potassium loss from isolated frog sartorius muscle. For frog liver and kidney slices, 20 to 22 mM of potassium per liter were needed in the incubating solution for maintenance of normal tissue potassium content. Steinbach (5) was able to soak potassium out of frog muscle in a potassium-free Ringer's solution. The exchange of potassium was reversible if muscles did not lose more than about half of their original content. He concluded from other experiments (6) that some potassium is bound with a non-osmotic fraction of muscle. Fenn and Cobb (7) found that potassium moved into muscle from media with greater than normal concentrations of potassium. Conway (8) stated that potassium chloride caused swelling of muscle, just as urea does if this be substituted for sodium chloride in isotonic Ringer's solution. There was no swelling when the sodium was maintained at a normal concentration in spite of the addition of potassium chloride.

The experiments to be reported below were designed to determine the change produced in rat liver potassium incubated *in vitro* for various times up to 120 minutes, and to determine the effect of anoxia and fluoride on these changes.

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<sup>1</sup> Fellow of the Commonwealth Fund, 1948-49.

## EXPERIMENTAL PROCEDURE

*Incubation.* Male albino rats of approximately the same weights were fasted overnight for 18 hours, killed by a blow, and then exsanguinated. The liver was promptly delivered and slices approximately .5 mm. thick cut with a Stadie slicer. Usually, one liver slice was placed directly into each of several 25 ml. Erlenmeyer flasks containing 2 ml. of a bicarbonate-Ringer medium. However, when potassium-free medium was used, the slices were rinsed first in a Petri dish with the medium, then five slices were incubated in a single flask in 10 ml. of the medium. After 30 minutes, each slice was placed in a separate flask with 5 ml. of the potassium-free medium. This was done to maintain the potassium of the medium at a low value.

The flasks were closed with two-hole stoppers and placed in a special shaking rack in a constant temperature water bath at 38°. The flasks were connected in series with rubber tubing. Aeration was carried out with 5 per cent carbon dioxide and 95 per cent oxygen for 5 minutes. Anaerobic incubation was carried out by aerat-

TABLE 1. IONIC CONSTITUENTS OF VARIOUS INCUBATION MEDIA, EXPRESSED AS mM PER LITER OF SOLUTION

SOLUTION NO.	K	Na	Ca	Mg	Cl	HCO <sub>3</sub>
1	5	152	1	1	121	40
2	0	157	1	1	121	40
3	34	123	1	1	121	40
4	78	78	1	1	120	40
5	110	0	10	20	130	40
6	110	46	1	1	120	40
7	152	5	1	1	121	40

All the media contained .00075% phenol red, and 200 mg. glucose/100 ml.

ing with 5 per cent carbon dioxide and 95 per cent nitrogen. The gas was bubbled through water at the temperature of the bath in order to saturate the gas with water vapor before passing on to the flasks. The average time from death of the rat to the beginning of aeration of the flasks was 7 minutes. The aeration tubes were then clamped and the flasks shaken for lengths of time varying from 15 to 120 minutes. The slices were then removed and the fluid was centrifuged and the supernatant was used for potassium analysis. An unincubated piece of liver was also obtained each time for analysis. Care was taken not to contaminate this piece with any of the solution.

*Treatment of Slices.* In some experiments, the incubated slices were weighed on a torsion balance before placing them in the cups. At the end of the incubation period, the slices were removed from the fluid, blotted gently on filter paper and placed in weighed cups for drying and determination of their dry weight. The cups were then filled with ethyl ether. The ether was removed after approximately an hour by using a capillary pipette. Three portions of ethyl ether and three portions of petroleum ether were used to remove fat. The cups were again dried and weighed to obtain the fat-free weight of the slices. The contents of each cup were emptied into digestion tubes using three or four portions of water to rinse the cups. Two ml.



of 10 N sulfuric acid were added to each digestion tube. The contents were heated over a micro burner until fumes of sulfur trioxide appeared. A few drops of concentrated nitric acid were then added and the mixture boiled until it was clear and colorless. The digest was diluted to a specific volume, usually 50 ml. using distilled water and a standard lithium sulfate solution so that the final lithium concentration was 6 mm/l. The potassium and sodium concentrations were determined using a Perkin-Elmer flame photometer (no. 52a). The concentrations are reported in terms of mm per liter of medium of mm per 100 grams of fat-free solids of liver.

*Media Used.* The ionic constituents of various media used are listed in table 1. All media contained 200 mg. glucose/100 ml. except where otherwise specified. *Solution 1* is the standard bicarbonate-Ringer solution used in most of the experiments. All solutions were saturated with either 5 per cent CO<sub>2</sub>—95 per cent O<sub>2</sub> or 5 per cent

TABLE 2. EFFECTS OF INHIBITION FOR VARYING LENGTHS OF TIME ON CONCENTRATIONS OF LIVER POTASSIUM AND SODIUM, IN mM PER 100 GRAMS OF FAT-FREE SOLIDS, AND CONCENTRATIONS OF POTASSIUM IN THE MEDIUM IN mM PER LITER OF SOLUTION<sup>1</sup>

ANALYSES		TIME OF INCUBATION IN MINUTES						
		0	15	30	45	60	90	120
Liver Potassium	Mean	31.8	18.5	22.8	25.2	26.2	27.2	26.9
	S.D.	1.6	1.7	1.6	1.9	2.4	3.0	2.3
	No. of det.	(42)	(10)	(16)	(12)	(16)	(13)	(9)
Liver Sodium	Mean	12.5	37.9	35.7	31.7	32.3	33.8	32.4
	S.D.	1.8	4.9	5.5	4.1	4.5	7.1	8.0
	No. of det.	(16)	(10)	(15)	(11)	(15)	(13)	(9)
Fluid Potassium	Mean	5.10	8.24	7.70	7.62	7.25	6.96	7.19
	S.D.		.44	.45	.72	.43	.64	.73
	No. of det.		(9)	(16)	(7)	(10)	(8)	(4)

<sup>1</sup> See table 1, *solution 1*.

CO<sub>2</sub>—95 per cent N<sub>2</sub>. The pH control was provided by the addition of phenol red to the incubating media and approximate colorimetric pH estimation. Occasional electrometric measurements of pH, using the Cambridge pH meter, showed the final pH's to vary from 7.42 to 7.53.

In certain experiments, the concentration of potassium was varied from 0 to 152 mm/l. as indicated in table 1 (*solutions 2-7*). *Solution 5* is one which Teng *et al.* (9) have found particularly suitable for the synthesis of glycogen by rat liver slices.

## RESULTS

The results to be presented will be grouped as follows: 1) the change in tissue sodium and potassium upon incubation of the liver slices in standard bicarbonate-Ringer solution for varying lengths of time (table 2); 2) the effect of varying the initial potassium concentration of the medium on the tissue potassium (fig. 1); and 3) the effect of sodium fluoride and a varying degree of anaerobiosis on the tissue potassium (table 3).

*Control Analyses on Fresh Liver.* The potassium and sodium concentrations in liver slices are expressed as mm/100 gm. of fat-free solids. The mean potassium concentration was 31.8 mm/100 gm. fat-free solids and the mean sodium concentration was 12.5 mm/100 gm. of fat-free solids. These correspond to 89.6 mm potassium and 34.8 mm sodium/kg. of fat-free tissue, values which correspond favorably with those previously published (10-12). In the experiments reported here, the control values serve as a basis for comparison of concentrations after different times of incubation. The percentage of water in the fresh liver was constant and averaged 71.9 gm/100 gm. of fat-free tissue.

*Equilibration in Standard Bicarbonate-Ringer Solution.* When solution 1 ( $K = 5$ ,  $Na = 152$ ) was used, there was an initial loss of potassium to approximately 60 per cent of the control value in 15 minutes (table 2). With continued incubation, there followed a return to about 80 per cent of the control value by 45 minutes, after which the tissue potassium remained constant. From the table, it is also seen that the solution potassium concentration rose from 5.1 to 8.2 mm/l. during the

TABLE 3. EFFECT OF ANAEROBIOSIS AND SODIUM FLUORIDE ON LIVER POTASSIUM

SERIES	CONDITIONS	NO. OF EXPER.	TIME OF INCUBATION IN MINUTES					
			15	30	45	60	90	120
1	5% CO <sub>2</sub> -95% O <sub>2</sub>	9	18.5	22.8	25.2	26.2	27.2	26.9
2	5% CO <sub>2</sub> -95% O <sub>2</sub> + NaF 16 mm/l.	2		11.1	9.7	10.3	12.0	12.5
3	5% CO <sub>2</sub> -95% N <sub>2</sub>	6	12.1	12.6		9.3	9.7	
4	5% CO <sub>2</sub> -95% N <sub>2</sub> for 5 min.	2	15.7	28.8	25.0	25.5		
5	5% CO <sub>2</sub> -95% N <sub>2</sub> for 10 min.	1	16.4	20.2	21.0	21.5		
6	5% CO <sub>2</sub> -95% N <sub>2</sub> for 15 min.	4	10.3	15.1	15.9	14.5		

Equilibrating solution 1.  $K = 5$  mm/l.,  $Na^+ = 152$  mm/l. Concentrations of potassium expressed as mm/100 gm. fat-free solids. Control liver potassium = 31.8 mm/100 gm. fat-free solids.

first 15 minutes, after which it declined to a concentration of about 7 mm/l. The changes in the potassium content of the medium are quantitatively accounted for by the changes in the potassium content of liver slices.

The changes in the tissue sodium upon incubation were in the opposite direction to the changes in tissue potassium. Furthermore, they were from 12 to 15 mm/100 gm. of fat-free solids greater than the changes in potassium. This may be interpreted as indicating not only an exchange of intracellular potassium for extracellular sodium but also about a 50 per cent increase in the extracellular fluid of the incubated tissue.

This series of experiments demonstrated that, whereas there is an initial rapid loss of potassium from the intracellular fluid of liver slices, this loss is to a very large extent reversible when the slices are subsequently well oxygenated. There remains, however, a portion of the liver tissue which still has less intracellular potassium and more intracellular sodium than has normal liver.

*Equilibration in Solution of Varying Potassium Concentrations.* The effects of varying the initial potassium concentration of the medium from 0 to 152 mm/l. are presented in figure 1. The results are expressed as mm of potassium/100 gm. of fat-free tissue solids against time. The final concentrations of potassium in the external media are designated on each curve.

It is seen that when the external medium initially had no potassium (final  $K = 0.4$  mM/l.), the tissue potassium fell from 32 to 10 mM/100 gm. of solids and remained there. When the initial potassium was 5 and 34 mM/l., respectively, the tissue potassium fell in the first 15 minutes, but with further incubation returned toward its initial value. With initial potassium concentrations of 78 mM/l. and higher, there was no initial drop in tissue potassium, but rather an increase proportional to the potassium in the incubating solution.

From these results, the following conclusions may be drawn: 1) that, with essentially no potassium in the external fluid, the tissue potassium drops markedly but, nevertheless, retains about 30 per cent of its original potassium; 2) that a potassium concentration of 7.5 mM/l., which is 50 per cent higher than that found in normal extracellular fluids, is not sufficient to maintain the normal tissue potassium of liver slices incubated *in vitro*; 3) that, under aerobic conditions, potassium concentrations above 36 mM/l. would appear to be sufficient to maintain the tissue potassium at approximately its normal value.

*Effect of Sodium Fluoride and Anaerobiosis.* Because of the rapid initial drop in tissue potassium observed in the previous experiments with bicarbonate-Ringer solution as incubating medium and the subsequent partial recovery when incubation was conducted under aerobic conditions, it seemed important to explore the effect of inhibiting aerobic metabolism by sodium fluoride and anaerobiosis. The results of these experiments are presented in table 3. All incubations were conducted in solution 1.

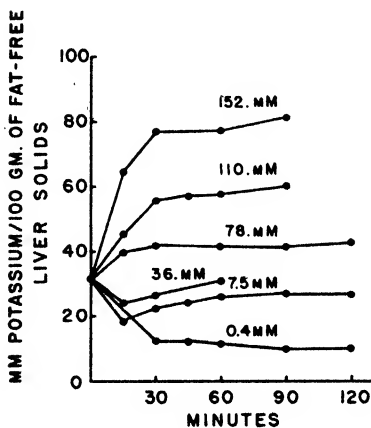


Fig. 1.

It will be seen that the addition of sodium fluoride in a concentration of 16 mM/l. caused a prompt decrease in tissue potassium to about 10 mM/100-gm. solids, the minimum residual value observed when no potassium was present in the outside fluid (*series 2*). There was no recovery of tissue potassium upon continued incubation. This demonstrates that the maintenance of the normal tissue potassium depends on normal aerobic metabolism of the liver.

A series of experiments were then carried out to follow the effect of incubation of the slices in 5 per cent  $CO_2$ —95 per cent  $N_2$  for varying lengths of time. When the entire period of incubation was conducted anaerobically (*series 3*), the results were essentially the same as those with sodium fluoride—i.e. the liver potassium dropped promptly from 32 to 12 mM/100 gm. of solids and remained at a value of about 10 mM throughout the 90-minute period of incubation. There was no evidence of a return toward normal as was found when the same experiment was carried out under aerobic conditions.

When periods of preliminary incubation in 5 per cent  $CO_2$ —95 per cent  $N_2$  of 5, 10 and 15 minutes preceded subsequent equilibration in 5 per cent  $CO_2$ —95

per cent  $O_2$ , the results recorded in *series 4, 5 and 6*, respectively, were obtained. It is seen that 5 minutes of anaerobic equilibration did not interfere appreciably with the subsequent recovery of the liver potassium, but 15 minutes of preliminary anaerobic equilibration interfered materially with recovery. One experiment at 10 minutes gave an intermediate result.

These experiments suggest that the loss of intracellular potassium by liver slices is markedly enhanced by interference with aerobic metabolism, but that the loss of potassium resulting from anaerobiosis of not more than 5 minutes is to a large extent reversible.

#### DISCUSSION

It seems reasonable to ascribe the initial fall of liver potassium concentration on incubation to the injury caused by slicing and the anoxia lasting from the time of cessation of circulation in the animal to the immersion of the slices in fluid equilibrated with oxygen and carbon dioxide. The results of early experiments in which the potassium loss was greatest and the time of preparation was the longest suggested to us that anoxia and trauma are important factors. The results of anaerobic incubation demonstrate that anoxia is indeed an important cause of the initial loss of potassium.

A rise in serum potassium has been observed in the intact animal which has been subjected to asphyxia and the excess potassium has been shown to arise chiefly from the liver (13). Dixon (14) observed no loss of potassium from brain slices if the medium contained glucose in spite of anaerobic incubation. The absence of glucose or the presence of sodium fluoride with glucose caused a loss of potassium from brain slices under the same conditions. On the other hand, Krebs and Eggleston (15) found an appreciable loss of potassium from brain slices even when glucose was present and the incubation was carried out aerobically. The addition of 5 mM/l. of L-glutamate prevented the potassium loss from brain slices. When we carried out similar experiments on liver slices, no demonstrable effect of either glutamate or glucose on tissue potassium was observed. These negative results are to be attributed to the rich store of potential substrates normally present in liver.

The ability of the liver to recover potassium from the medium against a considerable concentration gradient is a most striking finding and is indicative that metabolic work is being done in effecting the re-establishment of the intracellular-extracellular potassium gradient.

The present results illustrate that, although about 70 per cent of the intracellular potassium of the rat liver is quite labile and passes rapidly into extracellular fluids when the liver tissue is subjected to injury and anoxia, it is capable of being largely restored to its normal intracellular concentration if aerobic conditions are resumed and if a suitable concentration of potassium is maintained in the extracellular fluid.

The concentration of potassium in the incubating medium required to maintain a normal intracellular potassium concentration of about 175 mM/kg. of intracellular water would appear to be between 36 and 78 mM/l. With extracellular potassium concentrations less than 5 mM/l., the intracellular potassium decreased markedly,

and with extracellular potassium concentrations in excess of 80 mM/l., the tissue potassium increased in proportion to the increase in extracellular potassium concentration.

The excess of potassium, expressed as mM/100 gm. of fat-free tissue, above that of normal fresh liver reflects an increase in the potassium concentration of the extracellular fluid phase and an increase in the relative amount of extracellular fluid rather than an increase in the potassium concentration of the intracellular fluid above its normal value of 180 mM/kg. of intracellular water.

In view of the demonstrated influence of the potassium ion concentration on intracellular enzymic reactions, it is believed that the results reported above have significance for all metabolic studies in which tissue slices are incubated *in vitro*.

#### SUMMARY

When rat liver is sliced and incubated in a bicarbonate-Ringer medium, there is an initial loss of potassium of considerable magnitude from these slices, reaching a maximum about 15 minutes after slicing. There is a secondary rise of potassium concentration in the liver slices to a level approximately 80 per cent of the control. Concomitantly, there is a decrease in the fluid potassium concentration. When glycolysis is inhibited by sodium fluoride or when the tissue is injured by anaerobic incubation, there is a greater initial loss and a failure of the secondary rise of potassium to appear. An external potassium concentration of approximately 34 mM/l. causes a return of slice potassium at one hour to approximately the control level. Sodium concentrations are inversely related to the potassium concentrations.

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# FACTORS AFFECTING THE SYNTHESIS OF ACETYLCHOLINE BY BRAIN SLICES

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QUASTEL, Tennenbaum and Wheatley (1) and Stedman and Stedman (2) were the first to show that brain tissue, incubated aerobically in the presence of eserine as an inhibitor of cholinesterase, would synthesize acetylcholine (ACh) *in vitro*. Quastel *et al.* showed that the presence of an oxidizable substrate such as glucose was required. Mann, Tennenbaum and Quastel (3) differentiated two forms of ACh: a free, active, soluble form, and a pharmacologically inactive form which is attached to the tissue solids, and which can be broken down to the free form *in vitro* by the action of acids or certain organic solvents. Their experiments indicated that the inactive form is a precursor of the free form. They found that optimal synthesis was obtained in a bicarbonate-buffered Ringer-type medium in which the molarity of potassium ions had been increased to 27 mM, with a corresponding decrease in the sodium ion concentration to maintain isotonicity. The extra ACh was found entirely in the free form. They showed that this effect of high potassium could be inhibited by calcium or magnesium ions in 20-mM concentration. Finally, Mann *et al.* (3) reported that a reversible equilibrium was set up between free ACh in the medium and bound ACh in slices. They concluded that this was so since they found that a large proportion of the synthesis of free ACh took place in the first hour of incubation, and that subsequently there was little or no further synthesis. Also, in the presence of 27 mM potassium, they found that the addition of ACh to the medium caused inhibition of the synthesis.

The effect of high potassium on ACh synthesis by slices has been confirmed by Welsh and Hyde (4) though these authors obtained lower values than did Mann *et al.* In the following report the main observations of Mann *et al.* have been again confirmed, and many figures even higher than theirs have been obtained. However, confirmation of the existence of a reversible equilibrium between free and bound ACh could not be obtained. The rapid synthesis of ACh by slices has been shown to be dependent on a critical concentration of calcium ion and on the presence of bicarbonate, and to be inhibited by pyruvate or oxaloacetate when glucose is also present.

## METHODS

Hooded rats of a pure strain were used. They were killed by decapitation, and slices of cerebral cortex were prepared in a cool humid chamber, by means of a Stadie-

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Riggs microtome (5), without moistening, and weighed on a torsion balance. Usually about 80-mg. samples of tissue were introduced into Warburg vessels containing 2 ml. of medium. The vessels were filled with oxygen containing 5 per cent carbon dioxide when bicarbonate-containing medium was used, otherwise with pure oxygen, and were shaken in a bath at 38°C.

The normal, low potassium, bicarbonate-buffered, medium contained 122 mM NaCl, 3 mM KCl, 0.4 mM  $\text{KH}_2\text{PO}_4$ , 1.3 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 24.5 mM  $\text{NaHCO}_3$ , and 10 mM glucose. Any changes in concentration of any of the components of this medium were balanced by changes in the sodium chloride concentration to preserve isotonicity. Eserine sulfate, 0.4 mM was present in all media.

TABLE I. TIME COURSE OF ACh SYNTHESIS

INCUBATION TIME, HOURS				
0	1	2	3	4
<i>Low K<sup>+</sup>, 3 mM (Av. of 3 exper.)</i>				
4	10	100 (13 to 20)		
<i>High K<sup>+</sup>, 27 mM</i>				
1		100 (77)		164
4		100 (70)		171
7	32	100 (76)	171	
2	32	100 (65)	115	
	29	100 (70)		
	50	100 (40)		
		100 (60)		
	38	100 (55)	189	357
		100 (55)		346
	30	100 (69)		173
		100 (58)	141	155
				157
Av.	35	100 (63)	154	218

The amounts of free ACh found in the medium, per unit weight of tissue, are expressed as percentages of the amount found at 2 hours in each experiment. Absolute amounts of ACh,  $\mu\text{g/gm.}$ , are given in parentheses.

At the end of the experimental period, the suspending medium was separated from the slices by rapid filtration through filter paper discs and assayed directly for free ACh. For the determination of total ACh, 0.1 ml. of N HCl was added to the vessel contents bringing the pH to about 2.5, the slices were ground with sand and the suspension was allowed to stand for an hour, centrifuged, and shortly before assay, neutralized. For the separate determination of bound ACh, the medium was filtered off, the slices were ground in acidified medium and the suspension treated as for total ACh determinations. Mann, Tennenbaum and Quastel (3) showed that this treatment was sufficient to liberate all the bound ACh in the tissue. ACh was assayed on the eserinated leech muscle according to the procedure of Quastel, Tennenbaum and Wheatley (1) except that the wash fluid contained 0.01 mM eserine. The sensitivity of the muscle at the time of each assay was estimated as described by Elliott,

Swank and Henderson (6). Figures given usually represent the average of three assays.

### RESULTS

The rates of ACh synthesis obtained with similarly treated brain slices from different animals varied very widely; amounts of free ACh between 18 and 70  $\mu\text{g/gm.}$  have been obtained in one hour in the presence of high potassium. For this reason the effects of various factors have been compared only with controls carried out simultaneously on samples from the same brains and rates are given in the tables as percentages of the relevant control rates. No reason for the variability has yet been detected. Duplicate runs on samples of tissue from the same brain agreed within less than 10 per cent.

TABLE 2. EFFECT OF ADDED ACh—INCUBATION TIME 1 HOUR

ADDED ACh	FINAL ACh CONCENTRATION <sup>1</sup> IN MEDIUM	ACh (TOTAL) <sup>2</sup> LESS ADDITION	ADDED ACh	FINAL ACh CONCENTRATION <sup>1</sup> IN MEDIUM	ACh (TOTAL) <sup>2</sup> LESS ADDITION
$\mu\text{g/ml.}$	$\mu\text{g/ml.}$	$\mu\text{g/gm.}$	$\mu\text{g/ml.}$	$\mu\text{g/ml.}$	$\mu\text{g/gm.}$
<i>Low K<sup>+</sup>, 3 mM</i>			<i>High K<sup>+</sup>, 27 mM</i>		
	0.15	15		0.8	33
1.25	1.5	18	0.5	1.0	31
	0.4	20	1.0	1.7	39
1.25	1.8	29		0.7	31
	0.4	21	1.25	2.1	45
1.25	2.1	40		0.8	29
			1.25	1.9	31
				0.9	29
			1.25	1.5	29

<sup>1</sup> Since free ACh was not determined separately in most of these experiments, the concentrations of ACh in the medium were estimated from the total ACh found less the approximate amount of bound ACh in the tissue.

<sup>2</sup> This figure includes bound ACh originally present in the tissue.

*Time Course of Synthesis.* In table 1 are presented the results of experiments on the time course of the synthesis of free ACh in bicarbonate-buffered medium with low (3.4 mM) and high (27 mM) potassium ion concentrations. In confirmation of the work of Mann *et al.* (3), the high potassium concentration was found greatly to increase the synthesis of free ACh. There was relatively little increase in bound ACh. The results were very variable but there was no evidence that an equilibrium concentration of free ACh is reached. The production of free ACh was actually more rapid during the second than during the first hour and in three cases the rate increased further during the third or fourth hour. Results of experiments in which ACh was added to the medium at the beginning of incubation are shown in table 2. No inhibition of the synthesis was found, but occasionally an acceleration. Acceleration of the ACh synthesis by minced mouse brain in the presence of ACh has been reported by



Hobbiger and Werner (7), and Bülbring and Burn (8) find that ACh addition stimulates ACh synthesis by acetone powders prepared from rabbit auricles which have stopped beating.

Mann *et al.* (3) found less tissue-bound ACh at the end of experiments with high potassium than without. This effect was not obvious in these experiments. The effect of incubation on the bound ACh content of brain slices will be discussed further in later communications.

**Calcium and Magnesium Effects.** Mann *et al.* (3) found that, with the usual low potassium concentration, about the same rate of synthesis occurred in medium con-

TABLE 3. EFFECTS OF  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , AND  $\text{CO}_2$ -BICARBONATE

BUFFER		A	B	C	D	E	F	G	H
		No. of comparisons							
		7	3	12	3	2	4	6	4
		Modified Ringer-glucose medium containing 27 mM K <sup>+</sup> and buffers shown							
Bicarbonate 25 mM	Complete	100	100	100	100	100	100	100	100
	No Ca			30		39	30		
	No Mg					86			
	No Ca or Mg					27			
Bicarbonate 25 mM and Phosphate 20 mM	Complete				57				67
	No Ca				26				
Phosphate 20 mM	Complete	52	44						55
	No Ca		18				16		
No buffer	Complete							29	
Sheep serum plus 27 mM K <sup>+</sup> , and 4.5 mM added glucose									
Complete	In oxygen—5% CO <sub>2</sub>	100				100			
Decarbonated	In oxygen	42				42			

Average percentage of the synthesis of free ACh in complete bicarbonate-buffered medium in 1 hour.

taining phosphate or bicarbonate buffer, but that the stimulatory effect of high potassium was much less marked in phosphate than in bicarbonate. It seemed possible that this difference, which we have confirmed (table 3, col. A) might be due to lack of calcium ion in the phosphate medium. The calcium ion concentration in the normal bicarbonate medium was 1.3 mM, and although the calcium added to the phosphate medium was the same, the effective calcium ion concentration in the presence of 20 mM phosphate buffer may be as low as 0.15 mM (9).

Table 3 shows results of experiments in which calcium was omitted from both media (cols. B, C, D). It is evident that lack of calcium depresses synthesis markedly in both media. Omission of magnesium seems to depress synthesis slightly (col. E). Figure 1 shows the results of experiments in bicarbonate-buffered medium containing

varying concentrations of calcium. Maximum synthesis occurs at a calcium concentration of about 1.3 mM, which is the concentration of ionized calcium found in the cerebrospinal fluid and other extracellular fluids of the body. The finding of Mann *et al.* (3) that high, 20-mM, calcium concentration strongly inhibits the synthesis in the presence of high potassium has been confirmed, but it is also clear that calcium ion in low concentration is essential for maximal ACh synthesis by brain slices.

*Carbon Dioxide-Bicarbonate Effects.* The rate of synthesis is still considerably greater in bicarbonate than in phosphate-buffered medium even when neither contains any added calcium (table 3, *col. F*). Immobilization of calcium ion cannot therefore account for the whole difference between the two media. That the carbon dioxide-bicarbonate system is also essential for maximal synthesis is shown by the following observations.

In medium containing calcium but neither phosphate nor bicarbonate, the pH being carefully adjusted to equal that of the other media, the synthesis was consider-

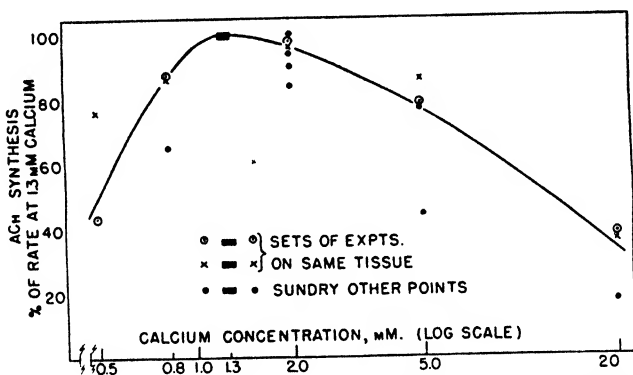


Fig. 1. EFFECT OF CALCIUM CONCENTRATION ON ACh synthesis by brain slices in Ringer-bicarbonate-glucose medium containing 27 mM.  $K^+$ .

ably lower than in the calcium and bicarbonate-containing medium (table 3, *col. G*). Synthesis in phosphate medium to which calcium was added was usually increased when bicarbonate was also present though not to the same level as in the presence of bicarbonate and calcium without phosphate since phosphate tends to precipitate the calcium (*col. H*). Finally, in sheep serum, in which the potassium concentration had been increased to 27 mM, synthesis was much diminished if the bicarbonate was first removed by acidification to pH 5.5, evacuation and readjustment to pH 7.4. The calcium content of the normal and decarbonated serum would be the same.

Results of experiments in which the bicarbonate and  $CO_2$  concentrations were varied are shown in table 4. Maximum synthesis occurred at about pH 7.4 in the presence of 5 per cent carbon dioxide and 25 mM bicarbonate. Any variation from these conditions depressed the synthesis but variability was too great to allow any conclusion regarding the effects of the individual factors.

*Effects of Oxaloacetate, Pyruvate and Citrate.* The possibility that  $CO_2$  exerts its effect through combination with pyruvate to give oxaloacetate was tested by adding

oxaloacetate to the phosphate or unbuffered media. Little or no effect on the synthesis was found. As is shown in table 5, oxaloacetate added to bicarbonate medium inhibited the synthesis markedly. Quastel *et al.* (1, 3) showed that pyruvate could replace glucose as substrate for brain slices synthesizing ACh, and we have confirmed this. But pyruvate added in addition to glucose causes inhibition. Adding citrate to calcium-containing media strongly inhibits the synthesis of ACh.

#### DISCUSSION

While most recent work on the acetylcholine synthesizing mechanism has been done on ground up tissue, dried powders or extracts, the present studies have been

TABLE 4. EFFECTS OF CARBON DIOXIDE TENSION AND PH

PH (CALCULATED)	6.77	7.07	7.37	7.67	7.97
CO <sub>2</sub> in gas mixture, 2.5%			(12.5 mm) 51A 97D 82E	(25 mm) 63A 59D 77E	(50 mm) 49A
5%		(12.5 mm) 60A 80B 42D 67E	(25 mm) 100 A, B, C, D, E	(50 mm) 46A 57D 51E	
10%	(12.5 mm) 39A	(25 mm) 34A 57B 44D 51E	(50 mm) 36A 43B 67C		

Modified Ringer-glucose containing 27 mM K<sup>+</sup> and varying concentrations of bicarbonate. Incubation time 1 hr.

Figures are percentages of maximum synthesis of free ACh. Letters indicate figures obtained in same set. Figures in parentheses give the bicarbonate concentration in the medium.

made on slices in order to obtain information of more direct physiological interest. Most of the results considered here have been obtained in the presence of the high, 27-mM, potassium concentration which stimulates maximal synthesis by slices (3). The intracellular potassium concentration is higher than this, but there is no clear evidence that high concentrations occur extracellularly though it is likely that they do so locally.

Though we have found no evidence that there is an equilibrium between bound and free ACh in brain tissue, we do not feel that the results reported exclude the possibility that reversal of liberation may occur to some extent under circumstances where free ACh is not diluted by diffusion from the site of its liberation. Hobbiger and Werner (7) have reported evidence for the attainment of such an equilibrium in fine suspensions of mouse brain. Studies by Abdon and Bjarke (10) indicate that, in

intact frog muscle, ACh which is liberated on stimulation is rapidly resynthesized into 'ACh precursor,' a soluble, pharmacologically inactive complex. Similar resynthesis seemed to occur in rabbit heart (11).

It is well known (12) that nervous structures show hyperirritability or spontaneous activity when deprived of calcium ion. In the present work, low concentrations of  $\text{Ca}^{++}$  have been found to be essential for maximal rate of ACh synthesis. This could be adduced as evidence that the ACh system is not essential to nervous activity. But there are several other possibilities. Bathing nerves or perfusing ganglia with calcium-free or even citrate-containing media may remove inhibitory amounts

TABLE 5. EFFECTS OF OXALOACETATE, PYRUVATE AND CITRATE

	NO ADDITION	OXALOACETATE 20 mM	PYRUVATE 20 mM	CITRATE 20 mM
Phosphate	13 14 17	17 16		6
No buffer	8 12 27?	11 9 33?		
Bicarbonate	59 61 46 38 49	27 14 35 16	17 28 24	8 7
Bicarbonate No Ca	9 12	4	5	3 8
Phosphate No Ca	6			6

Modified Ringer-glucose medium containing 27 mM  $\text{K}^+$  and buffer indicated. Media contained calcium, 1.3 mM, except where indicated.

Free ACh,  $\mu\text{g/gm.}$  found after 1 hour incubation.

of calcium which are somehow bound to the tissue structure leaving only the amount necessary for maximum synthesis. Or the rate of ACh synthesis may not be the factor which limits nervous activity but rather the ease of liberation from the bound form.

Strong inhibition of ACh synthesis by 9 to 35 mM  $\text{Ca}^{++}$  has been observed by Feldberg (13) with dried brain preparations but no requirement for a low concentration of calcium has been shown with cell-free preparations. Citrate strongly inhibits synthesis by brain slices, presumably chiefly by immobilizing calcium. With cell-free preparations from mammalian brain, citrate has a marked stimulatory effect on ACh synthesis (14, 15), but, with a highly purified system from squid ganglia, an inhibition has been observed (16). These varying results have not been explained but it seems probable that calcium ion may exert one type of effect with slices through its

action on cell membranes and a second type of effect on the enzyme system. Citrate may exert its effects through de-ionization of calcium and in some other manner.

There have been a number of reports that tissue respiration and glycolysis may be accelerated by the bicarbonate buffer system. These have been summarized and criticized by Birmingham and Elliott (17) who found only small, inhibitory effects with brain. As far as we are aware the effect here reported of carbon dioxide-bicarbonate in accelerating ACh synthesis is the only other case in which such an effect has been observed on a metabolic system which does not apparently involve carbon dioxide fixation. Though no comparison of buffers has been reported, bicarbonate-free media have been used successfully in most studies on the choline acetylase system in cell-free preparations. It seems likely that the effect of carbon dioxide-bicarbonate may be observed only with integrated tissue and may be connected with the permeability of cell membranes to carbonic acid. In extreme conditions the carbon dioxide tension of the plasma can vary between about 2 and 13 per cent of an atmosphere and the bicarbonate concentration between about 10 and 35 mM. Smaller variations than these could apparently exert an appreciable effect on ACh synthesis.

Though pyruvate can serve as substrate for respiration and concomitant ACh synthesis by tissue slices, it is interesting to find that when glucose is provided, the addition of pyruvate or oxaloacetate is inhibitory. Inhibitory effects of  $\alpha$ -ketoacids have been observed by Nachmansohn and John (14) with cell-free preparations from rat brain but not with the purified system from squid ganglia (16). The minimum concentration at which the inhibition is apparent with slices has not been studied but the full effect has been obtained with 10 mM added pyruvate. It seems possible therefore that  $\alpha$ -ketoacids may play a significant role in the regulation of the system *in vivo*. The mechanism of the inhibition is not understood but it is unlikely to be due to immobilization of calcium ion since pyruvate and various dicarboxylic acids do not de-ionize calcium salts as strongly as does citrate (18).

#### SUMMARY

The acceleration of the synthesis of ACh by rat brain slices by high potassium concentration has been confirmed. The rate tends to increase with time at first and synthesis continues at a high but variable rate for several hours. Addition of ACh to the medium does not depress, but sometimes increases, the rate of synthesis. In the presence of high  $K^+$  concentration, lack of  $Ca^{++}$  markedly inhibits ACh synthesis. Maximal synthesis occurs at about 1.3 mM  $Ca^{++}$ , and further increase in  $Ca^{++}$  concentration is inhibitory.  $Mg^{++}$  has similar but much smaller effects. The carbon dioxide-bicarbonate buffer system is required for maximal synthesis. Any deviation from normal plasma concentrations of  $CO_2$ , bicarbonate, or hydrogen ion, depresses synthesis. Pyruvate, oxaloacetate, and citrate, strongly inhibit synthesis in a glucose-containing medium.

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# COMBUSTION OF C<sup>14</sup> LABELED METHANOL IN INTACT RAT AND ITS ISOLATED TISSUES<sup>1</sup>

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FOR many years it has been recognized that methanol in contrast to ethanol can be only slowly metabolized by the mammalian organism, a significant percentage of ingested alcohol being excreted unchanged through the lungs and kidneys (1). It has naturally been supposed that the bio-degradation of methanol might follow the sequence of reactions expected on strictly chemical grounds, that is; methanol to formaldehyde to formate to carbon dioxide. Many workers have demonstrated an accumulation of formic acid in both blood and urine following methanol administration. Appreciable formaldehyde formation has not been found but this compound is logically assumed to be an intermediate between methanol and formic acid and to be oxidized to formate as rapidly as formed. Until recent availability of isotope derivatives the conversion of formate to carbon dioxide or the complete combustion of methanol could be inferred only from balance studies.

No details are available concerning the enzymatic mechanisms involved in the oxidation of methanol. Isolated liver alcohol oxidase will attack slowly the one carbon alcohol (2) and the tissue alcohol oxidases probably play a major role in the conversion to formaldehyde. Purified catalase is able to catalyze a hydrogen peroxide oxidation of alcohols (3) but it is unlikely that this mechanism operates in intact tissue. Formaldehyde might be oxidized to formate by liver aldehyde oxidase or by xanthine oxidase (4) or be changed to methanol plus formate by liver aldehyde mutase (5). There are a number of possibilities for the degradation of formate. It could be dismutated to carbon dioxide plus formaldehyde because of its potential aldehyde group, or split to carbon dioxide plus hydrogen, or dehydrogenated via one of the established electron transfer mechanisms to carbon dioxide and water, or tagged onto another compound which in turn would be oxidized in a manner analogous to acetate disposal, or even dehydrated to carbon monoxide plus water (carbon monoxide can be oxidized by higher animals to carbon dioxide) (6).

Methanol metabolism is of interest from the standpoint of the considerable number of severe poisonings and fatalities which have resulted from its consumption. Until very recently this alcohol and its oxidation products were thought to be substances completely foreign to the animal organism. It has now been demonstrated however that glycine (7), acetone (8) and methyl derivatives (9, 10) such as methionine and choline can act as sources of formate in the animal and that the formate

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can be used in the biosynthesis of serine and purine and also serve as a methylating agent, perhaps through reduction to methanol and formation of methyl phosphate (11). Therefore knowledge concerning the biological reaction of this alcohol assumes added interest and importance.

In the experiments to be presented  $C^{14}$  methanol has been used to help elucidate some of the details of methyl alcohol metabolism discussed above.

#### METHODS

The  $C^{14}$  radioactive methanol was prepared by reduction of carbon dioxide with lithium aluminum hydride (12). Because of the difficulty in removing some unidentified low boiling materials coming from the solvents, the alcohol was purified by crystallization of the 3,5-dinitrobenzoate followed by hydrolysis and recovery of the methanol. The specific activity was  $1.54 \times 10^5$  counts/minute/millimole.

A 200-gm. female rat was given by stomach tube 2.0 ml. of a 10-per cent radio-methanol solution (1 gm./kg.) containing 965,000 counts/minute. Immediately after administration of alcohol the animal was placed in a metabolism cage for collection of excretory products (13). Carbon dioxide and other expired gases were collected every 2 hours in 50 ml. of 2 N NaOH kept in an ice bath. At the end of the experiment the animal was sacrificed with ether, blood drawn from the dorsal aorta and the animal perfused with isotonic saline in order to remove most of the blood from the tissue. The individual organs plus the blood, a portion of the leg and back muscle and a sample of the visceral fat were frozen in dry ice and dried by lyophilization. These tissues were used to determine the extent of 'fixation' of the radioactive carbon.

Urine and the alkali solution containing the expired components were assayed for methanol, formaldehyde and formic acid. All chemical analyses were made by the chromotropic-formaldehyde colorimetric reaction (14); formaldehyde directly, methanol after oxidation with acid permanganate (15) and formic acid after reduction with Mg (16). Rats not treated with methanol gave no blank values for any of these substances. Radioactivity of the carbon dioxide exhaled is of course a measure of the complete combustion of the tagged methanol ingested by the rat. Aliquots of the alkaline solution containing the expired carbon dioxide were converted to barium carbonate and worked up for radioactivity measurements (17).

Five rats have been subjected to approximately the same experimental procedure. The results have been essentially identical in all cases. The data for the last animal studied are presented here for we feel that they represent the best technique and the most accurate findings.

#### RESULTS

It can be seen from the figure that there was a constant rate of combustion of the methyl alcohol as measured by the radioactive  $CO_2$  produced. During this steady state 2.5 per cent of administered counts were expired per hour, a methanol combustion rate of 5 mg/hour or 25 mg/kg/hour. Our previous experiments have shown ethanol to be oxidized at a constant rate (in the same size rat) of 175 mg/kg/hour (17). The ethanol was therefore oxidized seven times as rapidly as the methanol.



The constant rate followed by a precipitous drop to a very low output of tagged  $\text{CO}_2$  is evidence that the oxidation capacity of the animal is not to any significant degree dependent on the blood alcohol level. Sixty per cent of the ingested methanol was directly oxidized to  $\text{CO}_2$ . The additional 5 per cent of tagged  $\text{CO}_2$  recovered in the last hours probably results from the turnover of fixed tissue carbon.

Fourteen per cent of the administered methanol was eliminated unchanged in the expired air. The rate of expiration of the alcohol decreased gradually during the experiment, stopped at the end of the constant oxidation period and so was apparently dependent on the alcohol concentration in the blood. Three per cent of the methanol was recovered as such in the urine.

It was at first thought possible that an appreciable quantity of formaldehyde might be blown off from the lungs, but numerous tests failed to disclose even a trace. If formaldehyde were to accumulate to any degree in the blood stream it still would not readily volatilize through the lungs since in dilute solutions it exists as the hydrate which has about the same vapor pressure as water (18). No formaldehyde was excreted in the urine.

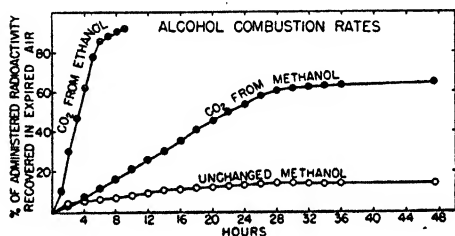


Fig. 1. RATES OF OXIDATION of methanol and ethanol in the rat. Excretion of unchanged methanol in expired air.

Formic acid was detected only in traces in the expired air but was excreted in the urine to the extent of 2.8 per cent expressed in terms of administered methanol. This urinary excretion was limited to the first 24 hours.

A portion of the methanol solution obtained from the expired gases was concentrated by fractional distillation, wet combusted to  $\text{CO}_2$  (19), and converted to barium carbonate for radioactivity assay. The carbonate had as expected essentially the same specific activity as the administered alcohol. It was remotely conceivable that endogenous methanol, formaldehyde or formic would appreciably dilute the activity. Less than 5 per cent dilution by such hypothetical intermediates would not have been detected by the procedure used.

The tissues which had been dried after sacrifice of the rat were powdered and assayed directly for radioactivity. Table 1 gives the results in counts per mg. of tissue and total counts in the individual organs. All the tissues except fat displayed an appreciable capacity for fixing methanol carbon, although there are quite large differences in their ability to do so. Those tissues with the most rapid synthetic activity and protein turnover, namely liver, kidney and intestine were the highest while brain, muscle and fat were low. With the data available it is impossible to determine how much of the tissue count was due to fixation of the carbon dioxide derived from the methanol. The total counts in all the tissues analyzed amounted to 4.0 per cent of the methanol given.

In order to obtain a preliminary indication as to which tissue components were fixed with radioactivity, the lyophilized liver was subjected to a separation into total fat, trichloroacetic acid soluble and 'protein' fractions. The finely powdered liver was thoroughly extracted in a Soxhlet with 1:1 petroleum ether—ethyl ether. The de-

TABLE 1. RADIOACTIVITY OF TISSUES FROM METHANOL-FED RAT

TISSUE	COUNTS/MG. × 10	COUNTS-TOTAL TISSUE	TISSUE	COUNTS/MG. × 10	COUNTS-TOTAL TISSUE
Liver	55	11,351	Blood	23	6,070
Intestine and stomach	53	7,325	Heart	19	294
Spleen	48	802	Brain	9	335
Kidney	41	1,285	Muscle	6	10,240
Lungs	33	562	Fat	0.5	

fatted meal was then exhaustively extracted with 5 per cent trichloroacetic acid. The activity in counts/mg. for the different fractions were: whole liver 5.5, protein residue 4.5, water soluble 6.1, fat soluble 5.2. The similar degree of activity for the different fractions indicates varied pathways in the body for methanol and its metabolites which is not unexpected in view of the reports on metabolic activities of formic acid.

TABLE 2. METABOLISM OF RADIOACTIVE METHANOL IN TISSUE SLICE

TISSUE	COUNTS/MIN/MG. OF TISSUE × 10	
	In respired CO <sub>2</sub>	Fixed in tissue
Liver	63.9	14.7
Kidney	21.4	1.5
Intestine	5.3	2.8
Heart	0.7	0.5
Diaphragm	0.6	0.7
Brain	0.03	0

To obtain a picture of the methanol oxidizing capacity of the individual organs the tissue slice technique was used. Tissue slices from various organs of a 200-gm. female rat were incubated in 50-ml. Erlenmeyer flasks equipped with a hanging center well for holding alkali. Each vessel contained 100 to 300 mg. (dry wt.) of tissue slice, 9.5 ml. of Ringer phosphate, 0.5 ml. of 0.4 M C<sup>14</sup> methanol and 1.0 ml. of 2 N carbonate free NaOH for absorption of the respiratory CO<sub>2</sub>. The vessels were gassed with oxygen in the cold, quickly brought to 38°C. and incubated with shaking for 2 hours. The C<sup>14</sup> methanol had the same specific activity as that fed to the rat in the previously described experiments. At the end of the incubation period the respired CO<sub>2</sub> was converted to BaCO<sub>3</sub> and assayed for radioactivity.

Table 2 summarizes the data on the tissue slice experiments. It can be seen that liver had the highest potentiality for burning methanol to CO<sub>2</sub>; about three times that of the kidney. The intestine was one-fourth as active as the kidney while diaphragm and heart were only about one-tenth that of intestine. Brain showed neglig-

ible activity. Of the tissue tested the liver has by far the greatest total organ oxidizing capacity for methanol. For comparative purposes the relative tissue slice capacities (per unit wt.) for combustion of ethyl alcohol were kidney 100, liver 35, heart 5, diaphragm 1 and brain 0. It is noteworthy that the liver and kidney activities are reversed as far as the oxidation of the two alcohols are concerned. Under the conditions of the incubation a fair amount of activity was fixed in the tissue components in the case of the liver. With the other tissue used only very small amounts were fixed.

#### SUMMARY

Methanol metabolism in the rat has been examined with the aid of the  $C^{14}$  labeled alcohol. Complete combustion as measured by radioactive  $CO_2$  formation proceeded independently of the alcohol concentration at a rate of 25 mg/kg. of rat/hour as compared to 175 mg/kg/hour for ethanol. Eighty-nine per cent of administered counts were recovered after 48 hours; 65 per cent as  $CO_2$  in expired air, 14 per cent as methanol in expired air, 3 per cent as methanol in urine, 3 per cent as formic acid in urine, and 4 per cent fixed in tissues. The relative methanol oxidizing capacities of rat tissue slice were liver 100, kidney 34, intestine 8, heart 1, diaphragm 1 and brain 0.

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# INHIBITION OF METHANOL OXIDATION BY ETHANOL IN THE RAT<sup>1</sup>

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ROE (1) has presented a detailed analysis of several cases of methanol poisoning, observing that those patients who had more or less simultaneously consumed ethanol showed less severe toxic symptoms. He advanced the hypothesis that ethanol competitively blocks the formation of methanol toxic oxidation products, presumably allowing a greater excretion of unchanged methanol, and on this basis he recommended ethanol therapy as an adjunct to the usual bicarbonate control of acidosis. Following this lead Zatman (2) in a note in the *Proceedings of the Biochemical Society* reported that ethanol inhibited the oxidation of the methyl analog by isolated liver alcohol dehydrogenase enzyme and also that in three human experiments ethanol increased the urinary excretion of methanol. Later Agner, Hook and von Porat (3) discussed two case histories of methanol poisoning wherein treatment with ethanol retarded methanol oxidation (fall of blood alcohol concentration).

In a preceding paper (4) we have examined the oxidative metabolism of methanol in the rat with the aid of the  $C^{14}$ -labeled compound. In an extension of this study the influence of ethanol on the oxidation of tagged methanol is here described and, as will be seen, more substantial experimental support is provided for Roe's conclusions. We have found ethanol to consistently depress methanol oxidation in rats. Two cases will be outlined in detail to give a picture of some of the effects which can be obtained.

## METHODS AND RESULTS

Rat 1 (fig. 1), a 200-gm. female, was given 1.0 gm/kg. by stomach tube of a 10 per cent radioactive methanol solution containing  $10^6$  counts. The animal was placed in a metabolism cage and hourly collections made of expired  $CO_2$  and methanol. The radioactivity of the respiration  $CO_2$  is a measure of the total combustion of ingested methanol. At the end of the 3rd hour and again after the 12th hour the rat received by stomach tube one ml. of 50 per cent (by vol.) ethanol. All techniques were the same as used in the previous papers (4, 5). The graph shows the striking block of methanol combustion which was produced each time by the ethanol and which was followed by a return to a normal methanol oxidation rate after a period approximating that required for the combustion of a major portion of the ethanol (5).

The second rat (fig. 2) presents a successful attempt to maintain methanol

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oxidation at a low level by continued administration of ethanol. A 200-gm. female rat was given one ml. of 50 per cent (by vol.) ethanol one-half hour before receiving the same dose of methanol given the first rat and placed in the metabolism cage for collection of expired gases. Every 4 hours, as indicated by the arrows in the figure, the animal received an additional dose of one ml. of 50 per cent ethanol. On the second evening dosing with ethanol was stopped and a total overnight collection of  $\text{CO}_2$  made. It is apparent that saturation of the animal with ethanol in large measure prevented the oxidation of methanol. In this experiment 30 per cent of administered counts were combusted to  $\text{CO}_2$  and 24 per cent appeared as methanol in the expired air. This

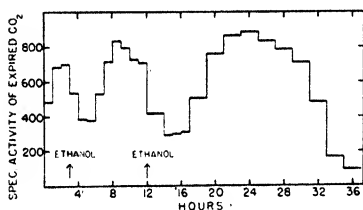


Fig. 1. OXIDATION OF METHANOL in the rat and influence of 2 doses of ethanol. Expired  $\text{CO}_2$  collected every one or two hours and specific activity recorded as counts per saturated planchet of barium carbonate. At 0 time rat given by stomach tube 2 ml. of an aqueous solution containing 200 mg. of radioactive methanol. At times marked by arrows the rat received by stomach tube 1.0 ml. of an aqueous solution containing 400 mg. of ethanol.

Fig. 2. OXIDATION OF METHANOL in the rat and influence of continuous administrations of ethanol. Two hundred mg. of radioactive methanol given at 0 time and 400 mg. of ethanol at times designated by arrows. Specific activity of expired  $\text{CO}_2$  recorded as counts per saturated planchet of barium carbonate. Upper curve, control with methanol alone.

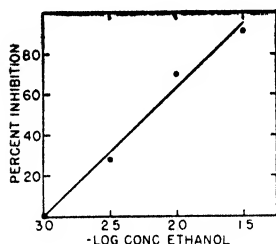
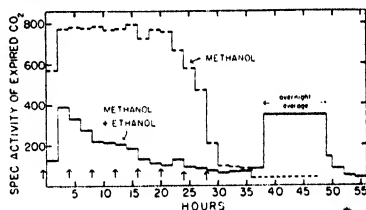


Fig. 3. INFLUENCE OF ETHANOL on oxidation of methanol by rat liver slice. Inhibition is expressed as percentage of radioactive  $\text{CO}_2$  produced in the presence of ethanol as compared with methanol alone.

compares with 65 per cent in the  $\text{CO}_2$  and 15 per cent as methanol in the expired air with the control rat receiving methanol alone (4). Undoubtedly a larger percentage of the methanol would have been eliminated unchanged if the ethanol treatment had been continued longer.

Figure 3 presents data for the effect of ethanol on methanol oxidation by liver slice. Two hundred to 300 mg. of tissue slice were incubated for 2 hours under oxygen in 10 ml. of pH 7.4 Ringer phosphate containing .01 molar  $\text{C}^{14}$ -labeled methanol and varying concentrations of ethanol, the flasks having separate cups with 1.0 ml. of 2N NaOH for collecting respired  $\text{CO}_2$ . The radioactivity of the  $\text{CO}_2$  again was used to measure the rate of methanol oxidation. It can be seen that added ethanol depressed

methanol combustion as a straight line function of the log of the ethanol concentration, a 72 per cent inhibition resulting at .01 molar. It is evident that the type of ethanol-methanol competition found in the intact animal holds true also for the isolated liver tissue.

It seems likely that the mechanism of the reaction herein described involves a competition between the alcohols for the first oxidative activating protein, the alcohol dehydrogenase, as indicated in the note by Zatman (2). We have here then another interesting example of a structural type of biological competition. Aside from the basic interest in the capacity of the organism to metabolize various compounds and their competition for the combustive pathways, the data provides an experimental background indicating the value of further clinical trials on the treatment of methanol poisoning with ethanol.

#### SUMMARY

Using the conversion of  $C^{14}$ -labeled methanol to radioactive  $CO_2$  as the test system, it has been demonstrated for the rat that ethanol produces a very considerable depression of the oxidation of methanol both in the intact animal and in liver slice.

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# EFFECT OF BARBITURATE ANESTHESIA ON POTASSIUM METABOLISM OF THE RABBIT AND DOG

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**D**URING recent studies on the relation of the adrenal cortex to potassium metabolism, it became apparent that more needed to be known about the effect of anesthesia on potassium metabolism in the experimental animal. Many observers have reported a lowering of serum K in dogs (1, 2), cats (3, 4), humans (5), and rabbits (6) during anesthesia. That anesthesia may influence urine formation has also been shown. Ether diminishes urine output in humans, rabbits and dogs (5); and barbiturates and chloralose depress urine output in dogs (5, 7). This depression may occur without a fall in systemic blood pressure (7), although either a fall in blood pressure or asphyxia may lead to oliguria (5). Lambrechts (7) found the sodium, chloride and phosphorus excretion diminished in dogs under chloralose anesthesia, but found the potassium excretion to remain the same or to increase slightly. While other observers (5) have confirmed the depression in excretion of most electrolytes under anesthesia, little is found in the literature concerning the effect of anesthesia on potassium excretion.

The purpose of the studies presented here was to observe in dogs and rabbits the effect of sodium pentobarbital (Nembutal) and Dial<sup>1</sup> on the plasma K level and urinary excretion of potassium. The effects of anesthesia on the distribution of intraperitoneally injected potassium were also studied.

## METHODS

White male rabbits, 4 to 7 months of age and 1.9 to 3.2 kg. body weight, were maintained on Purina fox chow. Trained adult male mongrel dogs of 8.9 to 17.7 kg. body weight were maintained on a regular kennel diet consisting of hospital table scraps. Each animal was fasted for 15 to 24 hours prior to an experiment, but was allowed water *ad libitum* except during the experiment. When possible each animal was used as its own control. A 3- to 5-cc. blood sample was obtained from the marginal ear veins of the rabbits and external jugular veins of the dogs. It was placed in centrifuge tubes in which 1.0 mg. of heparin had been dried, and centrifuged at 3000 r.p.m. for 5 to 10 minutes. The plasma was immediately pipetted into another tube and later diluted for potassium determination by a lithium internal standard flame photometer.

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<sup>1</sup> Di-allyl-barbituric acid 10 per cent, urethane 40 per cent, monoethyl urea 40 per cent; kindly supplied by the Ciba Pharmaceutical Co.

Urine samples were collected by catheterization, the bladder rinsed with 5 to 10 cc. distilled water after the collection of each sample, and the washings added to the sample. In anesthetized animals the catheter was left in place during the entire experiment. In the unanesthetized animals a catheter was sometimes left in place

TABLE 1. PLASMA POTASSIUM LEVELS (MILLIGRAMS PER CENT)

EX- PER. NO.	ANIMAL NO.	AGE	WT.	CONTROL		MINUTES AFTER INJECTION											
				(1)	(2)	15	20	30	40	45	60	90	120	180	360		
Group 1: Rabbits—Nembutal Intraperitoneally																	
		months	kg.														
1	R-3	6	3.2	20.3	20.0	19.0		15.8			17.4		15.3	19.0	16.5		
2	R-2	4	2.2		20.3	18.0		19.4			21.2		18.0	19.0	17.0		
3	R-4	5	2.8	20.4	20.0	19.0		19.0			17.5		18.0		18.3		
Group 2: Rabbits—Dial Intraperitoneally																	
4	R-3	6	3.0	20.3	20.5	18.5		18.8			17.0		16.8	16.4	15.8		
5	R-2	4	2.5		25.3	24.3		26.0				24.3		23.4			
6	R-4	5	2.6		19.9	18.5		20.0			17.3		16.8	18.5	19.5		
Group 3: Dogs—Nembutal Intravenously																	
15	44-28		11.4	20.8	18.4	16.5		16.0			16.8		17.8	18.0	20.0		
16	44-28		10.7		17.3		16.9	17.8			19.5			20.8	22.0		
17	48-223		17.7	16.3	17.0		15.8	16.5			16.5		17.8	17.3	18.5		
Group 4: Dogs—Dial Intravenously and Intraperitoneally																	
18	46-221		8.9	18.3	18.5	18.5		17.0			15.8		17.0	18.5	17.8		
19	47-234		11.7		19.8	19.5		16.5			15.3		12.5	13.0	17.3		
Group 5: Rabbits—5% KCl Intraperitoneally, Unanesthetized																	
7	R-2	5	2.6		20.5		25.0	27.5			24.8	24.8	23.0	22.0	23.6		
8	R-3	7	3.1		20.5		20.3	22.5			23.5	24.3	25.3	24.3	23.3		
9	R-4	7	3.1		16.8		16.9		18.3		21.5	24.0	24.1	22.6	20.0		
10	R-5	5	2.6		15.5		16.0	16.8			18.5	24.3	24.8	22.4	21.0		
Group 6: Rabbits—5% KCl Intraperitoneally After Dial Intraperitoneally																	
11	R-5	6	2.7	17.6	15.0		35.5	35.0			34.5	44.5	(Heart blood)				
12	R-8	6	3.0	21.8	18.6			38.3			44.5	45.0	39.7	40.5	38.6		
13	R-10	4	1.9	20.5	16.6		42.5		50.0	(Heart blood)							
14	R-11	4	1.9	20.8	20.8		41.2	41.0			40.0	36.8	40.5	44.0			

Plasma levels of potassium in rabbits under Nembutal (*Group 1*) and Dial (*Group 2*) anesthesia; in dogs under Nembutal (*Group 3*) and Dial (*Group 4*) anesthesia; and in unanesthetized rabbits given KCl intraperitoneally (*Group 5*) and rabbits under dial anesthesia given KCl intraperitoneally (*Group 6*). See text for discussion.

for several hours, but rarely intermittent catheterizations were necessary, the animals being returned to metabolism cages between catheterizations. Each sample of urine was measured, filtered and then diluted for potassium determinations with the flame photometer.

*Group 1: Nembutal Anesthesia of 3 Rabbits.* After a 2-hour control period, 0.75



cc/kg. body weight of 6 per cent Nembutal was injected intraperitoneally. Plasma K determinations were made on blood samples drawn 0, 15, 30, 60, 120, 180 and 360 minutes. Urine samples were collected hourly for 3 hours and at 6 hours.

TABLE 2. CHANGES IN URINE VOLUME, URINARY POTASSIUM EXCRETION, AND POTASSIUM CLEARANCE IN RABBITS AND DOGS UNDER NEMBUTAL AND DIAL ANESTHESIA

	URINE VOL.	URINE K	URINE K CONC.	K CLEAR.	URINE VOL.	URINE K	URINE K CONC.	K CLEAR.	URINE VOL.	URINE K	URINE K CONC.	K CLEAR.
	cc/kg/hr.	mg/kg/hr.	mg. %	cc/min.	cc/kg/hr.	mg/kg/hr.	mg. %	cc/min.	cc/kg/hr.	mg/kg/hr.	mg. %	cc/min.
<i>Group 1: Nembutal i.p. in 3 rabbits</i>												
	<i>Exper. 1, R-3</i>				<i>Exper. 2, R-2</i>				<i>Exper. 3, R-4</i>			
2-Hour control anesthesia	2.50	5.75	230	1.52	3.75	9.41	251	1.70	1.32	4.75	354	1.10
1 Hour	1.81	5.13	283	1.57	1.73	5.14	298	0.96	0.64	3.39	528	0.84
2 Hours	0.60	4.08	685	1.33	1.23	5.23	426	0.95	0.46	2.82	608	0.74
3 Hours	0.63	4.50	720	1.41	1.23	4.73	385	0.94	0.71	3.96	555	1.02
4-6 Hours	1.09	6.01	548		1.18	2.31	196		0.89	4.11	460	
<i>Group 2: Dial i.p. in 3 rabbits</i>												
	<i>Exper. 4, R-3</i>				<i>Exper. 5, R-2</i>				<i>Exper. 6, R-4</i>			
2-Hour control anesthesia	1.42	4.28	302	1.05	2.16	7.1	328	1.17	2.60	6.23	240	1.36
1 Hour	0.33	2.12	636	0.59	0.60	1.61	270	0.27	0.76	3.39	441	0.78
2 Hours	0.83	2.95	354	0.87	0.20	1.76	880	0.31	1.60	3.26	203	0.83
3 Hours	1.43	2.53	178	0.76	0.28	4.12	1471	0.74	6.12	3.65	60	0.90
4-6 Hours	1.86	2.62	141		0.47	1.40	300		2.53	3.35	133	
<i>Group 3: Nembutal i.v. in 3 dogs</i>												
	<i>Exper. 15, Dog 44-28</i>				<i>Exper. 16, Dog 44-28</i>				<i>Exper. 17, Dog 48-223</i>			
2-Hour control anesthesia	0.53	0.94	194	1.08	0.22	1.10	510	1.15	0.17	0.51	303	0.91
1 Hour	0.70	1.51	215	1.70	0.23	0.90	387	0.90	0.14	0.53	378	0.96
2 Hours	0.66	0.95	144	1.04	0.28	1.30	463	1.10	0.25	1.35	531	2.32
3 Hours	0.44	0.54	140	0.58	0.39	2.48	631	2.05	0.25	2.10	824	3.53
4-6 Hours	0.33	0.42	127		0.23	1.65	709		0.27	1.36	498	
<i>Group 4: Dial i.v. and i.p. in 2 dogs</i>												
	<i>Exper. 18, Dog 46-221</i>				<i>Exper. 19, Dog 47-234</i>							
2-Hour control anesthesia	0.98	4.27	439	3.48	1.85	14.0	754	13.8				
1 Hour	0.31	1.83	586	1.56	0.99	7.9	799	8.68				
2 Hours	0.75	0.99	133	0.91	0.76	2.1	276	2.94				
3 Hours	1.29	2.07	161	1.74	0.81	1.7	208	2.57				
4-6 Hours	1.08	2.94	272		0.79	3.9	493					

*Group 2: Dial Anesthesia of 3 Rabbits.* Procedure as under *Group 1*, 0.75 cc/kg. body weight of 10 per cent Dial being injected intraperitoneally.

*Group 3: Nembutal Anesthesia of Dogs; 3 Experiments.* Procedure as under

**Group 1**, 0.43 cc/kg. body weight of 6 per cent Nembutal being injected slowly intravenously.

**Group 4: Dial Anesthesia of Dogs, 2 Experiments.** Procedure as under **Group 1**, 0.43 cc/kg. body weight of 10 per cent Dial being given intravenously to one dog (*exper. 18*), and 0.75 cc/kg. body weight being injected intraperitoneally into a second dog (*exper. 19*).

**Group 5: 5 Per cent KCl Solution Intraperitoneally in 4 Unanesthetized Rabbits.** After a 2-hour control period 300 mg. of KCl/kg. body weight was injected intraperitoneally in a 5 per cent solution. Blood samples were collected before and 20, 40, 60, 90, 120, 180 and 360 minutes after the KCl injection. Urine collections were made as under **Group 1**.

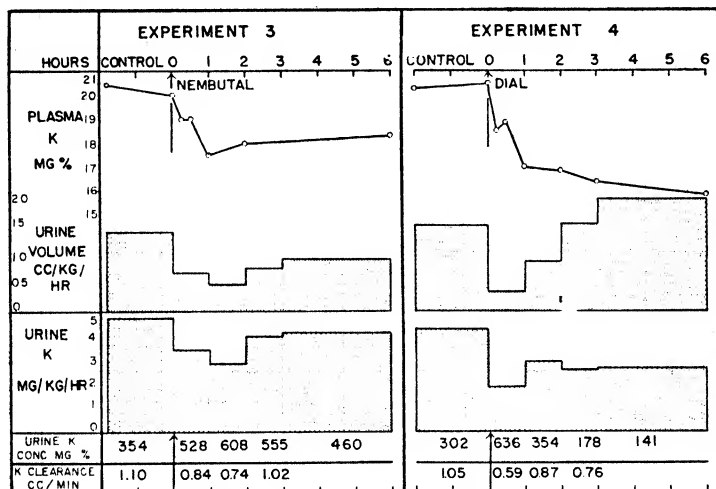


Fig. 1. *Experiment 3: PLASMA POTASSIUM and urine changes in rabbit 4 after intraperitoneal Nembutal anesthesia. Experiment 4 shows similar changes in rabbit 3 after intraperitoneal Dial anesthesia. See text for discussion.*

**Group 6: 5 Per Cent KCl Solution Intraperitoneally in 4 Rabbits Under Dial Anesthesia.** After a one-hour control period 0.75 cc/kg. body weight of Dial was injected intraperitoneally; and after approximately another hour's control period when the rabbit was well anesthetized, 300 mg. KCl/kg. body weight was injected intraperitoneally as a 5 per cent solution. Blood and urine samples were collected as for **Group 5**.

## RESULTS

**Effects of Nembutal and Dial on Rabbits: Groups 1 and 2.** In general, rabbits anesthetized with either Nembutal or Dial intraperitoneally remained quiet and well relaxed during the entire 6 hours of the experiment and were recovered fully by 18 hours.

The changes in plasma K levels are shown in table 1 and the changes in the

urine in table 2. A typical experiment from each group is graphed in figure 1. The responses to Nembutal and Dial were similar. There was a depression of plasma K level in all 6 rabbits within 15 minutes, a diminished urine output and potassium excretion and an increase in the concentration of potassium in the urine. The plasma clearance of potassium was likewise diminished. These changes associated with anesthesia were maximal during the first 3 hours at a time when the level of anesthesia was deepest as judged by eye signs and reflexes.

*Effects of Nembutal and Dial on Dogs; Groups 3 and 4.* In general, dogs under intravenous Nembutal anesthesia frequently trembled and began running movements during the second and third hour of anesthesia and by the end of 6 hours were sufficiently awake to drink water, although still moderately sedated. Under Dial intravenously or intraperitoneally, 2 dogs remained quiet for the full 6 hours.

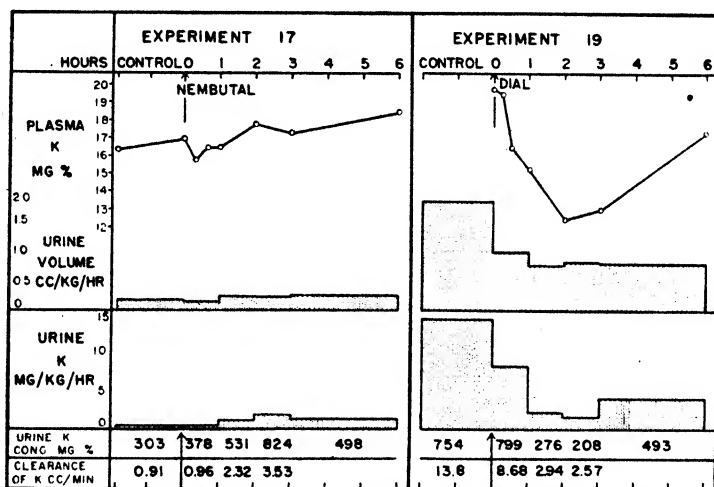


Fig. 2. *Experiment 17:* PLASMA AND URINE CHANGES in dog 48-223 after intravenous Nembutal anesthesia. *Experiment 19* shows changes in dog 47-234 after intraperitoneal Dial anesthesia. See text for discussion.

The changes in plasma K levels are shown in table 1 and urine changes in table 2. Typical experiments are graphed in figure 2. Intraperitoneal Dial (*exper. 19*) is seen to have a more marked and prolonged effect than a smaller amount of intravenous Dial (*exper. 18*). However, the results in both experiments are similar to those already presented in rabbits under Nembutal and Dial: i.e. a fall in the plasma K levels, and diminished urine output, potassium excretion, and plasma clearance of potassium. The concentration of potassium in the urine increased slightly during the first hour, then fell below the control levels.

Different results were obtained in 3 dogs under intravenous Nembutal anesthesia (*Group 3*). After a slight initial fall during the first hour, the plasma K rose above control levels for the remainder of the experiment. The urine changes were somewhat variable, but in general showed an increase in urine output, potassium

excretion, and urine potassium concentration. The plasma clearance of potassium increased during the first hour of one experiment and during the second and third hours in the other 2 experiments.

*Effect of Dial Anesthesia on the Distribution and Excretion of Intraperitoneally Injected Potassium in Rabbits; Groups 5 and 6.* The 4 unanesthetized control rabbits (Group 5) showed evidence of anxiety immediately following the intraperitoneal injection of KCl, but for the most part remained quiet on their backs for the remainder of the experiment. The animals of Group 6 were fully anesthetized when the KCl was injected intraperitoneally and remained quiet for the duration of the experiment. The plasma K changes are shown in table 1 and figure 3, the urine changes in table 3. Representative experiments are graphed in figure 4.

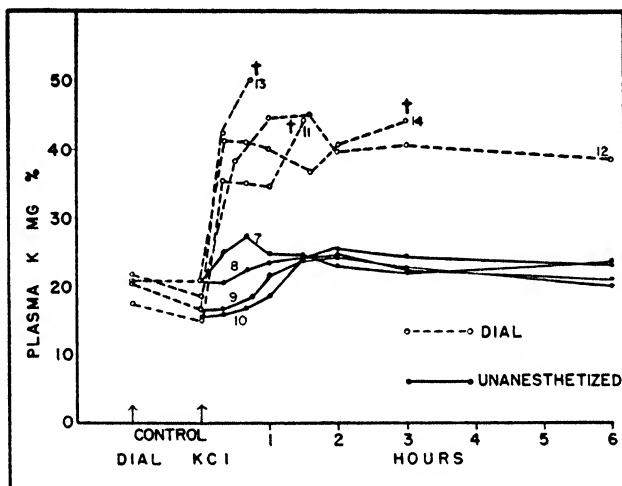


Fig. 3. EFFECT OF DIAL ANESTHESIA on plasma K levels after 5 per cent KCl intraperitoneally in rabbits. See text for discussion.

The unanesthetized control rabbits injected with 300 mg. KCl/kg. of body weight intraperitoneally showed a slow rise of plasma K to maximal levels of 123 to 160 per cent of control values and all 4 rabbits survived the experiment. The urinary output, urinary potassium excretion, urine potassium concentration and plasma K clearance were all increased for 6 hours, enabling an estimated 28 per cent of the injected potassium to be excreted in 3 hours and 54 per cent in 6 hours (average of 4 experiments). The volume of distribution of the injected potassium averaged 325 per cent of the body weight during the first 3 hours.

The identical amounts of KCl injected intraperitoneally into 4 rabbits under Dial anesthesia caused rapid and marked elevation of plasma K to toxic levels. This is in sharp contrast to the slow moderate rise in the unanesthetized rabbits (fig. 3). Three of the four rabbits died during the experiment and the fourth died shortly thereafter. One rabbit (*exper. 13*) died 45 minutes after the KCl injection, and during those 45 minutes showed marked diminution of urine and potassium

output. The other three rabbits, however, showed a marked increase in urine volume, urine potassium excretion, and plasma potassium clearance during the first hour to levels equal to or above those in the control rabbits. Subsequently these fell markedly to values far below the initial control values and far below those in the unanesthetized rabbits. The average excretion of injected potassium in 3 hours was estimated at 14 per cent, just one-half that in the unanesthetized rabbits. The volume of distribution of the injected potassium was only 57 per cent of the body weight, as compared to 325 per cent for the unanesthetized animals.

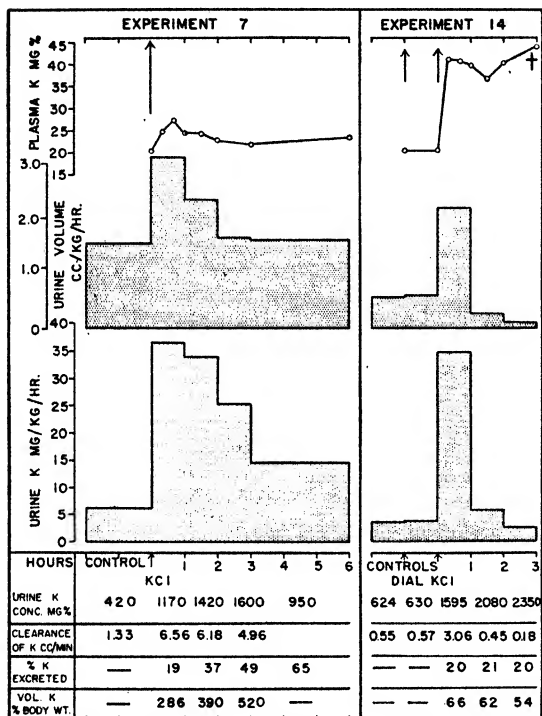


Fig. 4. *Experiment 7*: CHANGES IN PLASMA POTASSIUM and urine after 5 per cent KCl intraperitoneally in an unanesthetized rabbit. *Experiment 14* shows results of an identical injection of 5 per cent KCl in a rabbit under Dial anesthesia. See text for discussion.

## DISCUSSION

### *The Plasma Potassium Changes Under Anesthesia.*

The observed depression of plasma K in rabbits given Nembutal or Dial anesthesia and dogs given Dial anesthesia is in agreement with the observation of previous investigators (1, 2, 6). It has been shown that this same depression of plasma K occurs after nephrectomy (1). Our experiments confirm the fact that this fall in plasma K is not due to renal excretion of K, since the fall in plasma K occurred with a decreased urine excretion of potassium and a decreased plasma-renal potassium clearance. It has, therefore, been assumed that this potassium enters liver or muscle cells during anesthesia (1).

If this is the case, it may occur independently of adrenaline release with its subsequent deposition of glycogen and potassium in the liver cells since the fall in plasma K during anesthesia can occur after complete destruction of the adrenal medullae (2). However, in intact animals adrenaline action may contribute.

The direct effect of anesthesia on cellular metabolism has been studied indirectly and directly. General anesthesia depresses the general metabolic rate (2, 5) and 2-4 dinitrophenol, a metabolic stimulant, prevents the drop in plasma K after Nembutal anesthesia in dogs (2). Nahas (8) has demonstrated *in vitro* that

TABLE 3. CHANGES IN URINE VOLUME, URINARY POTASSIUM EXCRETION, POTASSIUM CLEARANCE; ESTIMATED PERCENTAGE INJECTED POTASSIUM EXCRETED AND VOLUME OF DISTRIBUTION OF INJECTED POTASSIUM IN RABBITS RECEIVING POTASSIUM CHLORIDE INTRAPERITONEALLY IN THE UNANESTHETIZED STATE (GROUP 5) AND UNDER DIAL ANESTHESIA (GROUP 6)

	URINE VOL.	URINE K	K CONC.	K CLEAR.	K EX-CRETED	VOL. K	URINE VOL.	URINE K	K CONC.	K CLEAR.	K EX-CRETED	VOL. K
	cc/kg/hr.	mg/kg/hr.	mg. %	cc/min.	% <sup>1</sup>	% <sup>2</sup> B.W.	cc/kg/hr.	mg/kg/hr.	mg. %	cc/min.	% <sup>1</sup>	% <sup>2</sup> B.W.
<i>Group 5: 5% KCl i.p. in unanesthetized rabbits</i>												
	<i>Exper. 7, R-2</i>						<i>Exper. 8, R-3</i>					
2 Hr. control	1.47	6.18	420	1.33			0.94	4.7	500	1.19		
KCl i.p.												
1 Hour	3.14	36.5	1170	6.56	19	286	1.25	10.1	810	2.42	3	304
2 Hour	2.35	33.9	1420	6.18	37	390	1.15	21.3	1825	4.46	14	282
3 Hour	1.58	25.3	1600	4.96	49	520	0.81	20.8	2580	4.34	24	314
4-6 Hour	1.53	14.5	950		65		1.03	17.6	1712		60	
	<i>Exper. 9, R-4</i>						<i>Exper. 10, R-5</i>					
2-Hr. control	0.92	4.0	434	1.23			0.65	4.0	617	1.12		
KCl i.p.												
1 Hour	1.26	5.3	421	1.49	1	330	0.56	4.3	761	1.09	0.2	520
2 Hour	1.50	17.9	1200	4.01	10	194	1.02	10.9	1063	2.08	5	162
3 Hour	2.29	32.3	1416	7.15	28	194	1.01	15.9	1568	2.88	12	200
4-6 Hour	1.61	15.1	937		64		0.76	12.0	1572		28	
<i>Group 6: 5% KCl i.p. in rabbits under Dial anesthesia</i>												
	<i>Exper. 11, R-5</i>						<i>Exper. 12, R-8</i>					
1-Hour control	3.48	3.8	110	0.97			0.32	3.0	926	0.68		
Dial												
1-Hour control	4.43	3.0	68	0.82			1.04	3.6	346	0.89		
KCl												
1 Hour	2.43	22.0	907	3.28	12	70	4.26	33.5	785	4.96	19	48
2 Hour	0	0					0.45	7.7	1721	0.87	22	58
3 Hour							0.63	6.6	1045	0.83	24	54
4-6 Hour							1.67	11.2	673		39	
	<i>Exper. 13, R-10</i>						<i>Exper. 14, R-11</i>					
1 Hour control	0.32	3.0	924	0.45			0.58	3.6	624	0.55		
Dial												
1-Hour control	0.28	2.4	855	0.41			0.60	3.8	630	0.57		
KCl												
1 Hour	0.13	1.0	790	0.09	0	48	2.20	34.9	1595	3.06	20	66
2 Hour							0.27	5.7	2080	0.45	21	62
3 Hour							0.10	2.4	2350	0.18	20	54
4-6 Hour												

<sup>1</sup> Percentage of injected K excreted, estimated from the K excreted above the control rate.

<sup>2</sup> Volume of distribution of injected K, expressed as percentage body weight.

Nembutal depresses the oxygen consumption by human erythrocytes. It is, therefore, possible that the entrance of potassium into tissue cells *in vivo* during anes-

thetia is related to a direct action of the anesthetic on cellular metabolism. An alternate possibility is that water leaves cells during anesthesia producing hemodilution. That such may occur has been shown by Adolph (9) in dogs under Amytal.

In *Group 3*, dogs under intravenous Nembutal, an initial slight fall with subsequent rise in plasma K levels may possibly be explained by the earlier recovery of these animals with subsequent trembling and running movements during the second and third hour of anesthesia, actions known to elevate plasma K (1, 10-12). Other investigators have found Nembutal to depress the plasma level in dogs (2).

*Urinary Changes under Anesthesia.* A transient oliguria corresponding to the period of deepest anesthesia was observed in all rabbits under Nembutal and Dial anesthesia. Anesthesia may result in the release of increased amounts of posterior pituitary antidiuretic hormone (13, 14) in dogs. That this oliguria may occur without change in systemic blood pressure has been shown in dogs (7). The diminished renal excretion of potassium with oliguria in the rabbits in *Groups 1* and *2* and the dogs in *Group 4* and the known depression of excretion of many electrolytes under anesthesia (5, 7) suggest a more general depression of renal function.

Emotional disturbances in the rabbit are known to cause an antidiuresis through diminished renal blood flow and filtration rate, the result of renal vasomotor changes rather than the pituitary antidiuretic principle (15). These renal vasomotor changes occur after adrenaline injection. Many of our experimental rabbits were used for repeated experiments and did not seem to be emotionally upset. A control experiment in which 10 cc. of isotonic saline was injected intraperitoneally and blood and urine changes followed for 3 hours, did not result in K shifts or oliguria. It is doubtful that the observed changes in blood and urine in these experiments could be explained on the basis of emotional disturbances. However, the oliguria and diminished potassium excretion under anesthesia may be the result of renal vasomotor changes similar to those due to emotion. If adrenaline were the mediator of the renal changes, it could also contribute to the plasma K depression.

In the experiments on dogs under Nembutal, the results were variable, but in two experiments the marked diuresis and increased potassium excretion occurred during the second and third hour after no significant change in the first hour of anesthesia. In the other experiments these changes were maximal during the first hour while the dog was relaxed. That species differences exist in the response to diuretic agents is known (16). In certain animals anesthesia may convert the antidiuretic response to posterior pituitary extract into a diuretic response.

*Effect of Dial on Distribution of Intraperitoneally Injected KCl.* If oliguria during anesthesia were primarily due to posterior pituitary antidiuretic action, we should still expect a diuresis after the administration of diuretic electrolytes, so long as renal function was not seriously impaired. Such was the case in two rabbits under Dial during the first hour after 5 per cent KCl intraperitoneally (table 3, *exper. 12* and *14*). The diuretic response, the increased excretion of potassium, and the increased plasma-renal potassium clearance in 3 of the anesthetized rabbits during the first hour equalled or surpassed those in the unanesthetized control animals receiving a similar KCl injection. The subsequent sharp fall in urine output and potassium excretion in the anesthetized rabbits occurred with a rise of plasma K to toxic

and lethal levels. These late changes may be the results of potassium toxicity on the heart and circulation rather than the effect of Dial *per se*. In one animal (table 3, *exper. 13*) there was no diuresis or increased potassium excretion in the first hour after KCl injection and death occurred after 45 minutes. In this animal more marked renal depression by Dial seems apparent.

The striking elevation of the plasma potassium to toxic and lethal levels in the anesthetized rabbits during the first hour after intraperitoneal potassium chloride (fig. 3) can not be explained by faulty renal excretion of potassium alone, since in 3 out of 4 experiments the potassium excretion was markedly increased during this first hour to a degree similar to the control *Group 5*. This rapid elevation of plasma K in the anesthetized rabbits stands in sharp contrast to the slow moderate rise of plasma K in the unanesthetized control rabbits. Likewise the volume of distribution of the injected potassium was at all times 5 to 7 times higher in the controls than in the anesthetized rabbits. It would thus appear that Dial either increased the rate of absorption of the potassium from the peritoneal cavity or slowed its entrance into the intracellular fluid. That it is all not simply due to a local effect of the intraperitoneally injected Dial on absorption of KCl by the peritoneal membranes is shown by an experiment in which the same results were obtained after *subcutaneous* Dial and intraperitoneal KCl. Further evidence against Dial's primary action being on the rate of absorption is unpublished data of Fenn *et al.* (17). In all rabbits autopsied 1 to 3 hours after a similar intraperitoneal injection of 5 per cent KCl, analysis of the peritoneal washings indicated a 90 to 95 per cent disappearance of the injected dose. It is conceivable that Dial might depress somewhat the peripheral circulation and the rate at which the injected potassium is distributed to the cells for uptake, or that it may act again directly on the cellular metabolism and cell permeability. There is insufficient evidence to draw final conclusions on this point, although the drop in plasma K during Dial anesthesia in *Group 2* does not suggest a decreased but rather an increased cell uptake of potassium.

In 4 experiments on dogs it was observed that an intravenous injection of 1 per cent KCl in doses of 15 mg/kg. body weight over 90 seconds resulted in similar transient plasma K elevations in both unanesthetized dogs and dogs under intravenous Nembutal anesthesia. In addition, both the anesthetized dogs excreted the injected potassium as rapidly or more so than they did when unanesthetized.

#### SUMMARY AND CONCLUSIONS

The effects of Nembutal and Dial anesthesia on the plasma K level, urine volume, K excretion, and K clearance in rabbits and dogs were studied for a 6-hour period. In 10 of 11 experiments the plasma concentration fell to values varying from 7.1 to 36.9 per cent below its control level. In one experiment on a dog under Nembutal, after an initial fall of 2.3 per cent there was a sustained rise of 20 per cent. In rabbits, both Nembutal and Dial anesthesia decreased the urine volume, total K excretion and K clearance, with an increased urine concentration during the first 3 hours. In dogs, Dial had similar effects, but Nembutal caused a minimal fall and a late rise of plasma K, a diuresis and increased K excretion, with perhaps an increased K clearance.



In rabbits the effect of Dial on the distribution of an intraperitoneal injection of 300 mg. KCl/kg. body weight was studied. Unanesthetized rabbits showed a slow rise in plasma K to an average maximum 140 per cent of control levels, and all 4 animals survived. The urine volume, K excretion, and K clearance were all markedly elevated, enabling approximately 28 per cent of the injected K to be excreted in 3 hours. Under Dial anesthesia, rabbits receiving identical amounts of intraperitoneal KCl showed an immediate, marked increase in plasma K to an average 263 per cent of control levels, and 3 of 4 animals died during the experiment. The urine volume, K excretion and K clearance increased during the first hour in a manner similar to the controls, but then fell rapidly to low levels so that only 14 per cent of the injected K was excreted in 3 hours. The marked elevation of the plasma K in the anesthetized rabbits was probably due to a diminished uptake of K by cells.

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# DISTRIBUTION OF PARENTERALLY ADMINISTERED LITHIUM IN PLASMA, BRAIN AND MUSCLE OF RATS<sup>1</sup>

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THE ingestion of LiCl by a normal human subject has been found to result in disturbances of muscular and nervous functions (1), and muscular weakness has been observed in rats injected with LiCl solutions (2). Severe derangements of nervous and muscular functions, in some cases resulting in death, have occurred in patients with cardiac insufficiency who have used LiCl as a salt substitute (3-6). Although the absorption and excretion of Li have been studied (7), little is known of the proportions in which Li is distributed between plasma and tissues, and a knowledge of this point appears to be essential for understanding the action of Li. For this reason, the investigations reported here were undertaken. Observations on the electroshock seizure thresholds of the experimental animals were included in an attempt to correlate brain excitability with the pattern of distribution of Li and Na between brain and plasma.

## METHODS

Male rats, obtained from the Sprague-Dawley farm at the age of 4 weeks, were kept in a constant temperature room at 27°C., and they were given free access to water and Purina Fox Chow. They were used for acute experiments when they weighed 250 to 320 gm. and for chronic experiments when their mean weight was approximately 275 gm.

For the chronic experiments a synthetic low Na diet was prepared. It had the following percentage composition: sucrose 73, casein 14, Crisco 8, cod liver oil 2, and low Na salt mixture 3. The salt mixture was prepared by omitting NaCl from the mixture described by Hubbel, Mendel and Wakeman (8). To each kilogram of diet were added the following amounts (in milligrams) of vitamins: thiamine hydrochloride 25, riboflavin 30, pyridoxine hydrochloride 25, calcium pantothenate 50, nicotinamide 100, inositol 100, *p*-aminobenzoic acid 600, and choline hydrochloride 500. Analysis of this diet showed that it contained 5.6 mEq. Na, 73.3 mEq. K and 69 mEq. Cl/kg.

In the acute experiments 0.15 N LiCl solution was injected intraperitoneally. The dose was 6 mm/kg. Fifteen minutes before and 25 minutes after the injection of the LiCl solution each rat was injected subcutaneously with 2 mg/kg. of atropine

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sulfate in 1 per cent solution. Controls of the acute experiments were treated with atropine sulfate only. In the chronic experiments no atropine was used, and one-half the daily dose of 0.15 N LiCl solution was injected subcutaneously at 8 A.M. and the remainder at 5 P.M. Blood and tissue samples were taken from these rats between 2 and 3 P.M. on the final day of the treatment.

TABLE 1. DISTRIBUTION OF ELECTROLYTES IN RAT PLASMA, BRAIN AND MUSCLE AFTER INTRA-PERITONEAL INJECTION OF LiCl SOLUTION, 6 MM/KG.

		NO.1	H <sub>2</sub> O	Li	Na	K	Cl
			gm.	mEq.	mEq.	mEq.	mEq.
Plasma	Controls	32	920 ± 1.1	<0.05	142.8 ± 0.33	4.7 ± 0.06	107.6 ± 0.33
	Hr. after						
	LiCl: 1	8	908 ± 1.0 (0.001)	7.6 ± 0.59	134.3 ± 0.73 (0.001)	5.3 ± 0.12 (0.001)	109.1 ± 0.77 (0.1)
	24	8	918 ± 0.4 (0.7)	2.0 ± 0.06	140.1 ± 0.23 (0.001)	4.6 ± 0.20 (0.7)	106.8 ± 0.73 (0.3)
	96	4	919 ± 1.8 (0.9)	<0.05	145.0 ± 0.71 (0.01)	4.3 ± 0.21 (0.1)	106.5 ± 1.55 (0.5)
Brain	Controls	18	792 ± 1.1	<0.05	49.9 ± 0.22	101.5 ± 0.43	34.1 ± 0.11
	Hr. after						
	LiCl: 1	8	786 ± 0.7 (0.001)	0.7 ± 0.06	48.7 ± 0.32 (0.01)	100.3 ± 1.02 (0.3)	34.1 ± 0.24 (0.9)
	24	8	790 ± 1.2 (0.2)	2.0 ± 1.6	46.2 ± 0.35 (0.001)	100.5 ± 0.82 (0.3)	31.7 ± 0.22 (0.001)
	96	4	793 ± 1.0 (0.3)	<0.05	49.4 ± 0.27 (0.2)	99.5 ± 1.71 (0.3)	33.9 ± 0.26 (0.4)
Muscle	Controls	8	773 ± 5.3	<0.05	21.6 ± 0.52	105.1 ± 2.17	14.9 ± 0.17
	Hr. after						
	LiCl: 1	8	760 ± 3.7 (0.1)	3.3 ± 0.18	20.3 ± 0.34 (0.05)	102.1 ± 2.42 (0.4)	16.0 ± 0.45 (0.05)
	24	8	771 ± 4.5 (0.8)	2.6 ± 0.15	21.2 ± 0.37 (0.5)	99.0 ± 1.52 (0.05)	14.6 ± 0.27 (0.4)
	96	4	773 ± 1.6 (0.9)	<0.05	21.8 ± 0.13 (0.7)	101.5 ± 0.87 (0.2)	15.8 ± 0.11 (0.01)

<sup>1</sup> Number of pooled samples; each pool consisting of tissue from a mean of 3 rats.

Figures in parentheses are *P* values of differences from controls.

Values/kg. wet weight; muscle values on fat-free basis.

Samples of blood and cerebral cortex were obtained by methods already described (9). No anesthesia was used, and the blood was handled anaerobically. Muscle samples consisted of the entire muscle mass surrounding the right femur. The blood and tissue samples of 2 to 5 rats were pooled. Water content was determined by drying the weighed tissue samples and aliquots of plasma at 105°C. to constant weight. The dried tissues were pulverized. Aliquots of tissue and plasma were ashed and dissolved according to the method of Hald (10), and the resulting solutions were analyzed for Na, K and Li by means of a Perkin-Elmer flame photometer (model 52A) used as a direct-reading instrument. In the analyses for Li

the unknown solutions were compared with standard solutions of LiCl containing appropriate amounts of NaCl and KCl. The determinations appeared to be entirely accurate with the exceptions that traces of Li (less than 0.05 mEq/kg. of tissue) could not be distinguished from absence of Li. Chloride was determined in plasma by the method of Schales and Schales (11) and in alkali-digested tissues (12) by the method of Van Slyke and Sendroy (13). The fat content of the muscle samples was determined by multiple extraction of separate aliquots with ethyl ether (14), and the values obtained were used in correcting those for water and electrolytes to a fat-free basis. No attempt was made to introduce a correction for blood content, since observations of Lowry and Hastings (14) on muscle and of this laboratory on brain indicate that in the exsanguinated rat the blood content of these excised tissues is negligible.

Electroshock seizure thresholds were determined by the standard method of this laboratory (9, 15, 16). The rats were thoroughly accustomed to the electroshock procedure before experiments were begun. In studying the acute effects of LiCl on brain excitability, seizure thresholds were determined one-hour and at 24-hour intervals after injection of LiCl. Atropine-treated controls were shocked at the same intervals. Observations on different groups of experimental and control rats were staggered in order to avoid too frequent shocking which in itself elevates the threshold (16). Seizure thresholds of the chronically injected rats were determined twice weekly between 2 and 3 P.M.

## RESULTS

In preliminary experiments it was found that oral or intraperitoneal administration of 0.15 N LiCl solution to rats not protected by atropine resulted in diarrhea. This was true in some degree of any dose larger than 1.0 mm/kg. Rats sacrificed one hour after the administration of LiCl showed marked hyperemia and hypermotility of the stomach and small intestine. The entire gastro-intestinal tract was distended with fluid. Blood collected from rats one hour after administration of 1.5 mm/kg. had a plasma Li level of 1.8 to 2.0 mEq/kg. It was necessary to control the diarrhea in order to prevent the loss of water and electrolytes and to permit the use of larger doses. This was done by the use of atropine as described above. Atropinized rats sacrificed one hour after the intraperitoneal injection of 6 mm/kg. of LiCl had gastro-intestinal tracts which may possibly have contained slightly more fluid than usual, but they were otherwise normal.

Table 1 shows the concentrations of water and electrolytes found in the plasma, brain cortex and muscle of control rats and rats given single intraperitoneal injections of 0.15 N LiCl solution in a dose of 6 mm/kg. Among the control samples all of the muscle and 8 of the plasma and brain samples were obtained from atropine-treated rats; the other control samples were from rats of the same age and size. The plasma and brain cortex of atropine-treated rats were not significantly different from those of normal rats, and the two types were grouped together.

One hour after treatment with LiCl the water concentrations of plasma, brain and muscle were reduced; at 24 and 96 hours they were normal. Li reached a level of 7.6 mEq/kg. in the plasma at one hour, had fallen to 2.0 at 24 hours and was

below detectable levels at 96 hours. Plasma Na changed inversely with Li so that the sum of the two was nearly constant, but at 96 hours the plasma Na was slightly elevated. Plasma K showed no significant change except a small elevation at one hour, and plasma Cl remained unchanged. In the cerebral cortex the Li concentration rose slowly and was accompanied by a loss of Na. At 96 hours no detectable amount of Li remained, and Na had returned to normal. There was no significant alteration in brain K, and the only change in Cl was the fall observed at 24 hours. In muscle the Li concentration rose much more rapidly than in brain. It was lower at 24 hours than at one hour, and it was not detectable at 96 hours. After a slight decrease at one hour, the muscle Na concentration returned to normal. There was some loss of K at 24 hours, and muscle Cl was slightly elevated at one and at 96 hours.

Table 2. FOOD CONSUMPTION, BODY WEIGHT AND SEIZURE THRESHOLD OF RATS SUBCUTANEOUSLY INJECTED WITH 0.6 mM LiCl/KG/DAY AND OF PAIR-FED CONTROLS

GROUP	4-DAY CONTROL PERIOD	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
<i>Mean food consumption in gm/rat/day</i>							
I	12.7	7.6	10.9	11.4	9.8	7.7	7.2
<i>Mean body weight in gm.</i>							
B	275 ± 3.5	267 ± 4.0	267 ± 4.0	280 ± 6.3	283 ± 7.7	279 ± 9.8	266 ± 14.9
C	275 ± 1.9	270 ± 2.4	268 ± 2.3	273 ± 3.3	285 ± 2.7	282 ± 2.8	275 ± 2.1
D	275 ± 3.9	273 ± 5.2	265 ± 4.4	270 ± 4.7	280 ± 3.9	277 ± 4.5	271 ± 4.8
<i>Mean electroshock threshold in ma.</i>							
B	22.7 ± 0.25	21.6 ± 0.22	20.6 ± 0.17	21.5 ± 0.22	20.9 ± 0.50	19.0 ± 0.66	17.9 ± 1.02
C	22.9 ± 0.43	22.2 ± 0.33	21.3 ± 0.53	21.7 ± 0.45	22.2 ± 0.33	21.9 ± 0.37	21.6 ± 0.36
D	22.5 ± 0.43	22.0 ± 0.44	21.0 ± 0.51	21.7 ± 0.43	22.6 ± 0.48	22.0 ± 0.68	22.6 ± 0.35

Group I: all groups. Group B: 0.6 mM LiCl/kg/day; Na intake <0.06 mEq/rat/day. Group C: controls pair-fed with group B; Na intake <0.06 mEq/rat/day. Group D: controls pair-fed with group B; Na intake 3.5 mEq/rat/day.

In the experiments on the effect of chronically administered LiCl two dose levels were used: 1.2 and 0.6 mM/kg/day. The solution was injected subcutaneously, and no diarrhea resulted. However, there was an immediate effect on the food intake. With the larger dose this effect was so great that it became necessary to stop the experiment on the 8th day. For 3 days before this the rats had refused food, and their mean body weight had decreased by 40 per cent. The effect of the smaller dose may be seen in table 2. There was a sharp decrease in food consumption during the 1st week, but by the 3rd week the intake was back to nearly 90 per cent of the normal level. Thereafter it declined progressively. This same pattern of food intake was observed in 2 other groups of rats injected with the same dose.

The data given in table 2 were obtained from 10 LiCl-injected rats and 20 others pair-fed with them on the same low Na diet. Of the latter group, 10 were given daily supplements of NaCl in the food. The amount of Na added was 3.5 mEq/rat/day, approximately equal to the daily intake of a 275-gm. rat eating Purina

Fox Chow in unrestricted amounts. The body weights of all the rats varied with the food intake.

Table 3 summarizes the concentrations of water and electrolytes found in the plasma and tissues of rats injected for 6 weeks with 0.6 mM LiCl/kg/day (*group B*), controls pair-fed the low-Na diet (*group C*) and pair-fed controls given a supplement of NaCl (*group D*). In addition a group of rats which received 1.2 mM LiCl/kg/day for 8 days is included (*group A*). These animals had a mean Li concentration of 0.9 mEq/kg. in plasma, 1.0 mEq/kg. in brain and 0.8 mEq/kg. in muscle. In comparison with normal controls (table 1) they showed significant decreases in plasma and brain Na and Cl. The decrease in muscle Na was of doubtful significance. *Group B*, after 6 weeks of treatment with LiCl, showed only slightly higher concentrations of Li than were found in *group A*. The decrease in plasma Na was of doubtful significance, but there were significant losses of Na from brain and muscle and of K from muscle. *Group C* showed an elevated plasma Na content accompanied by a loss of brain Na and of muscle K. *Group D* also had elevated plasma Na, but the brain and muscle Na remained within normal limits. In all of the groups there appeared to be a small decrease in brain water, and *group B* also showed a slight loss of plasma water.

Electroshock seizure thresholds were followed in both acutely and chronically treated rats. In 55 rats given a single intraperitoneal injection of 6 mM/kg. of LiCl in 0.15 N solution it was found that the seizure threshold showed the following changes from the pre-treatment level: at one hour, -17 per cent; at 24 hours, -16 per cent; at 48 hours, -3 per cent; at 72 hours, +3 per cent; at 96 hours, +6 per cent; thereafter up to 168 hours, no significant difference from the initial level. Of the changes observed only those at one and 24 hours are of true statistical significance. These changes were not caused by the atropine which the animals received, for 42 atropine-treated controls showed no alteration in seizure threshold. In rats injected daily with 0.6 mM LiCl/kg. the seizure threshold fell slightly during the first period of reduced food intake and much more precipitously during the last two weeks of the experiment (table 2). Both the low Na and the high Na control rats showed only small variations in seizure threshold.

#### DISCUSSION

Gastro-intestinal distress following relatively large intakes of LiCl has been reported in some human subjects (17). In others, it either fails to occur or is overshadowed by other symptoms referable to disturbances of nervous and muscular functions (3-6). In 1903 Good (18) reported that one of the principal effects of LiCl administered orally or subcutaneously to cats is the production of diarrhea, and he stated that in his experience a dose of 60 mg. (1.4 mM)/kg/day always kills cats and dogs sooner or later from gastroenteritis. Although his data are hard to evaluate, it appears from them that possibly cats are more and dogs less susceptible than rats to gastro-intestinal irritation produced by LiCl. A severe diarrhea was produced in rats by the intraperitoneal injection of 0.15 N LiCl solution in doses of 1.5 mM/kg. or greater, but no diarrhea was observed in those which received 1.1 mM/kg/day for 8 days by subcutaneous injection. The probable explanation of the

difference is that in the latter group, on account of the smaller divided doses and slower absorption, the plasma concentration of Li remained below the critical level for the production of diarrhea. The mechanism by which the irritation is produced is not evident, for the fact that it can be prevented by atropine indicates only that a cholinergic mechanism is involved.

Although rats which were treated chronically with 0.6 or 1.2 mM LiCl/kg/day failed to develop diarrhea during the time they were under observation, they showed a loss of appetite. This was not caused by the low Na diet which they received, for it has been found in this laboratory that adult rats fed this diet for 6 weeks gained weight slightly more rapidly than rats of the same age fed a stock diet (19). The

TABLE 3. CHRONIC EFFECTS OF LOW-SODIUM DIET AND SUBCUTANEOUS LiCl ADMINISTRATION ON ELECTROLYTES OF RAT PLASMA AND TISSUES

	GROUP	NO. <sup>1</sup>	H <sub>2</sub> O	Li	Na	K	Cl
			gm.	mEq.	mEq.	mEq.	mEq.
Plasma	A	6	919 ± 0.5	0.9 ± 0.02	140.3 ± 0.55	5.0 ± 0.12	104.3 ± 0.95
	B	13	914 ± 0.6	1.3 ± 0.08	141.2 ± 0.54	4.8 ± 0.12	106.5 ± 1.37
	C	6	917 ± 1.0	<0.05	147.7 ± 0.41	4.7 ± 0.17	107.5 ± 0.55
	D	8	918 ± 1.1	<0.05	148.4 ± 0.93	4.4 ± 0.12	108.0 ± 0.57
Brain	A	6	785 ± 2.1	1.0 ± 0.03	46.6 ± 0.72	101.2 ± 1.14	32.0 ± 0.24
	B	13	785 ± 0.3	1.1 ± 0.09	47.0 ± 0.33	100.5 ± 0.45	31.3 ± 0.49
	C	6	786 ± 1.2	<0.05	47.4 ± 0.37	102.0 ± 0.86	33.3 ± 0.33
	D	8	784 ± 0.4	<0.05	48.3 ± 0.63	103.4 ± 0.67	33.6 ± 0.32
Muscle	A	6	767 ± 1.7	0.8 ± 0.05	19.9 ± 0.53	102.3 ± 0.48	14.9 ± 0.36
	B	13	764 ± 2.5	1.1 ± 0.10	19.0 ± 0.60	93.6 ± 1.31	14.2 ± 0.22
	C	6	760 ± 7.2	<0.05	19.8 ± 0.60	90.5 ± 2.48	15.2 ± 0.70
	D	8	764 ± 2.8	<0.05	22.6 ± 0.62	96.6 ± 2.45	15.8 ± 0.42

<sup>1</sup> Number of pooled samples; each pool consisting of tissue from a mean of 3 rats.

Group A: 1.2 mM LiCl/kg/day for 8 days; Na intake <0.04 mEq/rat/day. Group B: 0.6 mM LiCl/kg/day for 32 to 42 days; Na intake <0.06 mEq/rat/day. Group C: Controls pair-fed with group B; Na intake <0.06 mEq/rat/day. Group D: Controls pair-fed with group B; Na intake 3.5 mEq/rat/day.

Values/kg. wet weight; muscle values on fat-free basis.

loss of appetite may have been produced by a direct effect of Li on the gastrointestinal tract or by the presence of low concentrations of Li in the central nervous system.

The data presented here suggest that Li, like many other ions and unionized substances, passes relatively slowly across the blood-brain barrier. In rats one hour after the injection of 6 mM LiCl/kg. the concentration of Li was 7.6 mEq/kg. in plasma, 3.3 mEq/kg. in muscle and only 0.7 mEq/kg. in brain. If it can be assumed that the Cl of brain is almost entirely extracellular, the formula of Hastings and Eichelberger can be applied (20), and it can be calculated that the concentration of Li in the extracellular water of brain was only 2.6 mEq/kg. or about 30 per cent that in plasma water. At the same time the concentration of Li in muscle was suf-

ficient to allow not only for equilibrium of the extracellular fluid with plasma but also for the entrance of 3.6 mEq/kg. of Li into the intracellular water. At 24 hours, when the plasma level of Li had fallen to 2.0 mEq/kg., the concentration in the total brain water exceeded that in plasma, suggesting that at this time a fraction of the Li was distributed in the intracellular phase of brain. Again assuming that Cl is extracellular in both muscle and brain, it can be calculated that at 24 hours the intracellular water of brain contained 2.8 mEq/kg. of Li while that of muscle contained 1.9 mEq/kg. Lithium appears not only to enter but also to leave the brain more slowly than it does the muscle.

In this connection it is of interest to examine the Li concentrations found in chronically treated rats. At the end of 6 weeks those rats which received 0.6 mM LiCl/kg/day had 1.3 mEq/kg. in the plasma and 1.1 mEq/kg. in both muscle and brain. These concentrations are approximately 1.4 mEq/kg. in the total water of plasma, brain and muscle. In any given 24-hour period the plasma concentration of Li must have varied rhythmically as the result of the absorption of LiCl after the 2 daily injections. The analyses were made on the tissues of rats sacrificed 6 to 7 hours after the last injection, and at that time the Li was apparently distributed uniformly among plasma water and the intra- and extracellular water of brain and muscle. In the rats which received 1.2 mM/kg/day there was undoubtedly a wider rhythmic variation in plasma Li on account of the larger amounts injected. These animals were sacrificed on the 8th day of treatment 6 to 7 hours after the last injection. It can be calculated that at the time when they were sacrificed the extracellular fluid contained slightly over 0.9 mEq/kg. of Li while the intracellular water of brain contained 1.27 and that of muscle 1.06 mEq/kg. This is probably a reflection of the higher plasma levels which occurred during absorption of LiCl and of the slow passage of Li out of the brain. In neither set of chronically treated rats is there evidence of accumulation of Li in cerebral or muscular tissue.

The fall in the electroshock seizure threshold of rats one hour after the intraperitoneal injection of 6 mM LiCl/kg. was accompanied by a corresponding decrease in plasma Na concentration. At this time the plasma, brain and muscle all showed slightly lower concentrations of water than were found in the controls. However, it can be calculated by the formula of Hastings and Eichelberger (20) that in spite of the loss of water the concentration of Na in the extracellular water was reduced by about 7 mEq/kg. The loss of Na from the extracellular fluid is probably the reason for the increased brain excitability at this time, for under a variety of experimental conditions in the rat there is an inverse relationship between plasma Na concentration and cerebral excitability (9, 21-23). Conversely, the small increase in extracellular Na concentration at 96 hours may explain the slightly elevated seizure threshold at this time. The low seizure threshold at 24 hours must be explained on some other basis, for although the threshold was as low as at one hour the extracellular Na had returned to within 2.5 mEq/kg. of the control value. At 24 hours the brain contained a higher concentration of Li than was found at one hour or in the chronically treated animals. It is possible that Li, either directly or through an effect on enzyme systems (2), may increase brain excitability.

In the experiments in which LiCl was administered chronically, not only the



presence of Li in the tissues but also the factors of low Na intake and restricted caloric intake must be considered. Rats injected daily for 6 weeks with 0.06 mM LiCl/kg. had significantly lower electroshock seizure thresholds than did rats pair-fed a diet low or high in Na. Both types of pair-fed controls had significantly elevated plasma Na concentrations without corresponding elevation of seizure thresholds, a finding which may be explained on the basis of chronic underfeeding (19, 24, 25). The lower seizure threshold of Li-treated rats may be related to the relatively lower plasma Na concentration, the presence of Li in the central nervous system or to both. The losses of brain Na and muscle K in the Li-treated rats were apparently a result of the chronic underfeeding and of the low Na intake.

#### SUMMARY

The intraperitoneal or oral administration of LiCl to rats in doses of 1.0 mM/kg. or greater resulted in diarrhea which could be prevented by atropine. Following the intraperitoneal administration of 6 mM LiCl/kg. the cerebral excitability of rats as measured by the electroshock seizure threshold was increased for at least 24 hours. The increased excitability appears to have been caused in part by the loss of plasma Na and in part by the presence of low concentrations of Li in the central nervous system.

Rats treated chronically with 0.6 mM LiCl/kg/day showed a loss of appetite in the first and again in the 5th and 6th weeks. They had lower seizure thresholds than did pair-fed controls and, when sacrificed at the end of 6 weeks, lower plasma Na concentrations. Although the situation was complicated by restricted food intake, the increased cerebral excitability again appears to be related to the loss of plasma Na combined with the presence of Li in the central nervous system. Calculations of the intra- and extracellular distribution of Li show that it passes more slowly into and out of cerebral tissue than muscle tissue. There was no indication of accumulation of Li in either tissue.

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# METABOLISM OF RAT HEART SLICES, WITH SPECIAL REFERENCE TO EFFECTS OF TEMPERATURE AND ANOXIA<sup>1</sup>

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THE low resistance of cardiac muscle to acute anoxia is indicated by the rapid loss of function following occlusion of the coronary circulation (1). It is also shown by the decrease in oxygen consumption of rat heart slices following anoxia induced by exposure of the animals to low oxygen tension (2) or hemorrhagic shock (3). The effects of the exposure of heart slices to complete anoxia and to various oxygen tensions *in vitro* are reported here. Until recently the few values for oxygen consumption and glycolysis of rat heart *in vitro* in the literature failed to show any general agreement as to the magnitude of these processes. In order to obtain uniform rates of oxygen consumption of rat heart slices, certain factors which contribute to the variation have also been studied. Particular attention has been paid to the effect of temperature<sup>2</sup>.

## METHODS

Adult albino rats of the Slonaker-Wistar strain were used. The heart was removed immediately after decapitation and transferred to a moist cold chamber maintained below 10°C. (4). Thin slices (0.2–0.3 mm.) were cut from the ventricular walls with a double-edge razor blade using the template technique of Crismon and Field (5). Although calculation from the Warburg formula (cf. 5) indicated that the limiting thickness for rat heart slices under these experimental conditions was about 0.57 mm., the thinner slices were used to insure adequate oxygen supply in the experiments at low oxygen tensions. Pearson *et al.* (6) found maximum rates of oxygen consumption with slices between 0.5 and 0.6 mm. in thickness.

Aerobic experiments were carried out in Ringer-phosphate-glucose solution of the following composition: NaCl 120 mM, KCl 2.4 mM, CaCl<sub>2</sub> 1.7 mM, MgCl<sub>2</sub> 0.8 mM, Na<sub>2</sub>HPO<sub>4</sub>·NaH<sub>2</sub>PO<sub>4</sub> 10 mM, glucose 11 mM, pH 7.35. Unless otherwise indicated the gas phase was oxygen. For determination of anaerobic glycolysis the solution contained 25 mM NaHCO<sub>3</sub> instead of the phosphate, and the gas phase was 5 per cent CO<sub>2</sub>–95 per cent O<sub>2</sub> (passed over hot copper).

The results of the manometric experiments are expressed as microliters of gas consumed or produced per milligram initial dry weight per hour ( $Q_{O_2}$  and  $Q_{O_2}^{N_2}$  re-

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<sup>2</sup> An abstract has appeared in *Federation Proc.* 3: 11, 1944.

spectively). Lactic acid was determined by the method of Barker and Summerson (7) on the entire contents of the vessels. The lactic acid production is expressed as microliters of lactic acid (as a gas) per milligram initial dry weight per hour ( $Q_{LA}^{O_2}$ ) and represents the difference between that in control vessels removed from the bath after thermoequilibration and that in vessels removed one hour later.

TABLE 1. EFFECT OF TEMPERATURE ON OXYGEN CONSUMPTION OF RAT HEART SLICES

Temp., °C.	10	15	20	25	30	35	37.7	40	42.5
No. of animals	3	3	3	3	7	5	27	3	3
Mean $Q_{O_2}$	0.08	2.09	3.26	5.14	7.22	8.31	9.97	8.55	4.54
S.E. of mean					0.48	0.39	0.41		

The medium was Ringer-phosphate-glucose. From 2 to 6 determinations were made on each animal.

The water content of heart slices from 9 animals was determined by drying to constant weight at 110°C. The mean percentage water was 75.5 with a S.E. of the mean of 0.25. The wet weight/dry weight ratio of 4.08 thus obtained was used to calculate the  $Q_{O_2}$  on a dry weight basis.

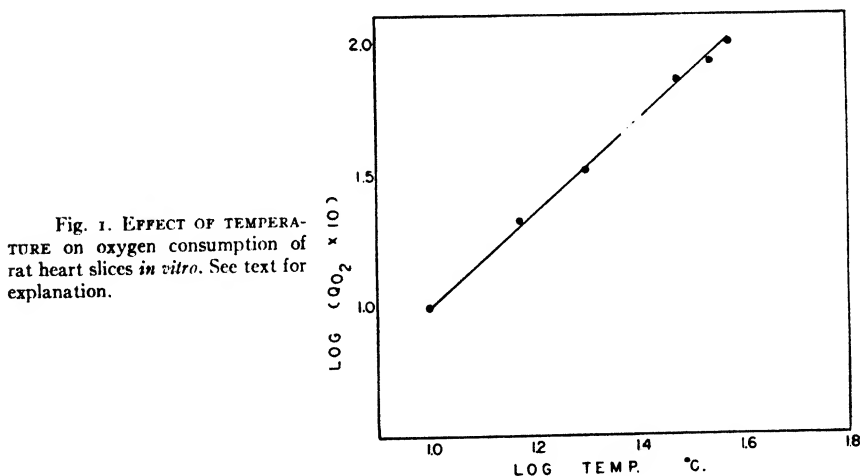


Fig. 1. EFFECT OF TEMPERATURE on oxygen consumption of rat heart slices *in vitro*. See text for explanation.

## RESULTS

*Factors Affecting Variability of  $Q_{O_2}$ .* Preliminary experiments indicated that the  $Q_{O_2}$  was higher in 100 per cent  $O_2$  than in air. Webb *et al.* (8) have reported similar results for the endogenous respiration of rat heart slices. Consequently all measurements of  $Q_{O_2}$  have been made with 100 per cent  $O_2$  as the gas phase. In agreement with Pearson *et al.* (6) we found higher rates of oxygen consumption when glucose was present in the medium from the beginning of the experiment than when the medium contained no glucose. Other investigators have failed to demonstrate such an effect when glucose was added after the start of a run (3, 9-11). In duplicate determinations on one animal the  $Q_{O_2}$  was higher at pH 7.4 than at pH 6.8 or 7.8 (cf. 8).

Since the  $Q_{O_2}$  was constant for at least 30 minutes, and usually for 40 to 60 minutes after thermoequilibration, the rates of oxygen consumption are calculated for this steady state period. Progressive decline in  $Q_{O_2}$ , similar to that reported by Pearson *et al.* (6), was observed at later time periods. The  $Q_{O_2}$  of rat auricles has been reported to be higher than that of the ventricle (12, 13). In order to determine whether or not there was a regional variation in the  $Q_{O_2}$  of ventricular tissue, the oxygen consumption of the right ventricle, left ventricle and apex was determined separately. The mean  $Q_{O_2}$  based on duplicate samples from 7 animals was: right ventricle 8.20, left ventricle 9.87, apex 9.38. The difference between the mean  $Q_{O_2}$  of the right and left ventricles was not significantly different ( $P > 0.9$ ). In subsequent experiments slices from the different regions were pooled before filling the vessels.

*Effect of Temperature on Oxygen Consumption.* The effect of temperature on the oxygen consumption of heart slices was determined by measurement of the  $Q_{O_2}$  at approximately 5°C. intervals between 10° and 42.5°C. The results are given in table 1. These results differ in certain respects from the results obtained with rat brain (14), kidney cortex (15), liver (16) or skeletal muscle (17). The optimum temper-

TABLE 2. GLYCOLYSIS IN RAT HEART SLICES

TEMPERATURE °C.	NO. OF ANIMALS	MEAN $Q_{O_2}^{N_2}$	S.E.	NO. OF ANIMALS	MEAN $Q_{O_2}^{LA}$	S.E.
37.7	9	1.36	0.13	6	0.29	0.27
0.3	3	0.53	0.06			

Aerobic: Ringer-phosphate-glucose; 100%  $O_2$ ; anaerobic: Ringer-bicarbonate-glucose; 5%  $CO_2$ -95%  $N_2$ .

ature for the oxygen consumption of rat heart slices is about 38°C. This is a lower optimum than that found for the other rat tissues. Furthermore it is found that for rat heart slices  $\log Q_{O_2}$  is a linear function of  $\log$  temperature in °C. over the range 10° to 37.7°C. (fig. 1). The relationship may be represented by the equation  $\log Q_{O_2} = 2.2088 + 1.777 \log t$ , in which  $t$  is temperature in °C. This is an example of the application of the general formula  $y = ax^b$ , suggested by Bělehrádek (18) as an empirical equation describing the variation of many biological processes with temperature.

For rat cerebral cortex, kidney cortex and skeletal muscle  $\log Q_{O_2}$  is an approximately linear function of temperature in °C. over a considerable temperature range, while for rat liver a graph of  $Q_{O_2}$  as a function of temperature yields a curve consisting of two linear segments.

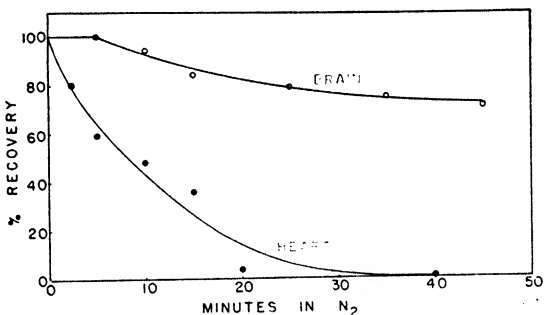
*Reversibility of Cold Inhibition of  $Q_{O_2}$ .* Fuhrman and Field (19) have shown that the rate of oxygen consumption of slices of rat cerebral cortex and kidney cortex returns to the initial level after periods of up to one hour at 0.2°C. Similar results have been obtained with slices of the myocardium. Since these experiments required a time longer than that during which the  $Q_{O_2}$  remained constant, the rates of the cooled tissues were compared with those of the controls at the same time after death

of the animal. When this was done it was found that  $Q_{O_2}$  of the cooled heart slices after being returned to  $37.7^\circ\text{C}$ . was higher than that of the controls for the same time period. The  $Q_{O_2}$  of the cooled tissue was 128 per cent, 111 per cent and 107 per cent of the controls after 45, 60 and 90 minutes respectively at  $0.3^\circ\text{C}$ . The absolute rates of oxygen consumption after cooling were of course lower than during the initial period at  $37.7^\circ\text{C}$ . This 'preservative' effect of cold is probably the result of a less rapid destruction of essential enzymes or co-enzymes at  $0.3^\circ\text{C}$ . than at  $37.7^\circ\text{C}$ .

**Glycolysis.** The results of the determination of anaerobic glycolysis (manometric) and aerobic lactic acid production (chemical) are given in table 2. The anaerobic glycolysis was a linear function of time for the one hour during which it was measured. In duplicate determinations on 2 animals the anaerobic lactic acid production, determined chemically, amounted to 114 per cent of total acid production determined manometrically. Wollenberger (20) has reported the  $Q_G^{N_2}$  of guinea pig heart slices to be of about the same magnitude as that found for the rat.

**Effect of Anoxia.** The effect of various periods of anoxia *in vitro* on the oxygen consumption of heart slices is shown in figure 2. The  $Q_{O_2}$  was determined in duplicate

Fig. 2. RECOVERY OF OXYGEN CONSUMPTION of rat heart and brain slices following anoxia *in vitro*. Recovery is expressed as percentage of that during an initial period in 100% oxygen, corrected for the decrease in  $Q_{O_2}$  with time of control slices from the same animal in 100% oxygen.



vessels for a 30-minute period. Nitrogen, passed over hot copper, was then introduced into the gas space of the vessels for the desired time while shaking was continued. This was then replaced with  $O_2$  and the  $Q_{O_2}$  again determined. For each animal 1 or 2 vessels were run for the entire time with oxygen as the gas phase. The recovery of oxygen consumption after anoxia is expressed as the percentage of that during the initial period, corrected for the decrease in  $Q_{O_2}$  of the oxygen control, for the same animal and the same time after death. For comparison with the effects of anoxia on the  $Q_{O_2}$  of heart slices, the effect of anoxia *in vitro* on the  $Q_{O_2}$  of rat brain slices is also shown in figure 2.

When rats were subjected to progressively decreasing atmospheric pressures until death occurred, the  $Q_{O_2}$  of slices of the heart was found to be markedly decreased (2). Heart slices taken from rats in the terminal stages of hemorrhagic shock also were found to have a  $Q_{O_2}$  below that of controls (3). Semi-anaerobic incubation of the whole rat heart *in vitro* (6, 9) or leaving it in the dead animal (8) also resulted in low rates of oxygen consumption. Webb *et al.* (8) have also reported that anoxia *in vitro* without glucose decreases the  $Q_{O_2}$  of heart slices determined in air.

The results reported here indicate the approximate time course of the effect of

anoxia on the oxygen consumption of heart slices. It may be seen from figure 2 that in our experiments 40 minutes of anoxia was sufficient to completely abolish the subsequent uptake of oxygen. The heart is more sensitive to the effects of anoxia under these experimental conditions than is the brain, liver, kidney or skeletal muscle.

In order to determine whether or not a similar decrease in oxygen consumption could be produced in heart slices by partial, rather than complete, anoxia, a second series of determinations was made. The capacity of the heart slices to consume oxygen was determined in 100 per cent oxygen after a constant exposure of 20 minutes to various oxygen tensions. The results are given in table 3. As in the experiments with total anoxia, the  $Q_{O_2}$  was first determined in 100 per cent  $O_2$ , then mixtures varying from 5 per cent to 50 per cent oxygen were introduced for 20 minutes, after which the  $Q_{O_2}$  was again determined in 100 per cent oxygen. As in the total anoxia experiments corrections were made for the decrease in  $Q_{O_2}$  of the controls. It may

TABLE 3. EFFECT OF PARTIAL ANOXIA ON OXYGEN CONSUMPTION

TEMPERATURE	OXYGEN	CONTROL $Q_{O_2}$	EXPERIMENTAL $Q_{O_2}$	RECOVERY
°C.	%	% of initial	% of initial	%
37.7	0	72.4	2.6	3.6
	5	82.0	33.3	40.6
	10	57.9	31.6	54.6
	15	70.0	41.1	58.7
	21 (air)	70.0	48.6	69.5
	50	68.0	57.7	85.0
15	10	68.0	56.0	82.3
	21 (air)	74.3	56.5	76.1

Medium: Ringer-phosphate-glucose:  $Q_{O_2}$  determined in 100%  $O_2$ . The figures are mean<sup>s</sup> of 2 or 4 determinations. All experimental vessels exposed to the given oxygen tension for 20 minutes.

be seen from the table that 50 per cent oxygen or less decreased the  $Q_{O_2}$  of the heart slices. This effect was less at 15°C. than at 37.7°C. Christensen and Pearson (21) have reported somewhat similar results.

#### DISCUSSION

It seems probable that the failure of heart slices to regain the original rate of oxygen consumption after brief periods of anoxia is due to destruction of one or more essential enzymes or co-enzymes during the period of anoxia. Preservation of such an enzyme at low temperatures would account for the higher  $Q_{O_2}$  following anoxia at 15° than at 37.7°C. Bernheim and Bernheim (9) found that anoxia did not depress the oxidation of lactic or pyruvic acids, and Govier (22) found that only moderate decreases in co-enzyme I and thiamine pyrophosphate occurred in the dog heart after one hour of anoxia. These data indicate that co-enzyme I, thiamine pyrophosphate or lactic or pyruvic dehydrogenases are not the substances involved.

## SUMMARY

Values are presented for the rates of oxygen consumption, anaerobic glycolysis and aerobic lactic acid production of rat heart ventricular slices with glucose substrate. The  $Q_{O_2}$  was determined over the temperature range  $10^\circ$  to  $42.5^\circ\text{C}$ . The optimum temperature was  $37.7^\circ\text{C}$ . Over the range  $10^\circ$  to  $37^\circ\text{C}$ .  $\log Q_{O_2}$  was a linear function of  $\log$  temperature in  $^\circ\text{C}$ . The oxygen consumption of rat heart slices was decreased by exposure *in vitro* to complete anoxia for periods of from 2.5 to 40 minutes. Exposure for 20 minutes to partial anoxia varying from 5 to 50 per cent oxygen also decreased the rate of oxygen consumption subsequently determined in 100 per cent oxygen.

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# EFFECT OF X-RAY IRRADIATION ON THE ALKALINE PHOSPHATASE OF THE PLASMA AND TISSUES OF RATS<sup>1</sup>

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**D**URING experiments designed to determine the effect of total body x-ray irradiation on the enzymes of rat plasma, it was found that the alkaline phosphatase activity was decreased. Representative tissues such as the thymus, spleen, liver and kidney were studied to determine if similar decreases in alkaline phosphatase could be observed.

## METHODS

Inbred male rats of Wistar strain, 60 to 70 days old and weighing between 150 and 200 gm. served as the experimental animals. The animals were fed a stock diet consisting of Gaines Dog Meal and Purina Checkers. Food was withdrawn the night before the animals were sacrificed; water was always available. In the pair-feeding experiments, the Gaines Dog Meal alone was fed. The conditions used for irradiation are described in a previous paper (1). Groups of animals were sacrificed at varying intervals after irradiation. The rats were anesthetized with sodium pentobarbital and were exsanguinated by drawing blood from the aorta into a syringe moistened with heparin. Liver, spleen, thymus and kidney were homogenized in a Waring Blendor with ice water.

The alkaline phosphatase activity of plasma and tissue homogenates was determined by the procedure of Binkley, Shank and Hoagland (2) with minor modifications. A buffer solution consisting of 20.5 gm. sodium barbiturate, 0.37 gm. barbituric acid and 2 gm.  $MgCl_2 \cdot 6 H_2O$  in one liter  $H_2O$  was used. The test plasma (one ml.) was diluted to 25 ml. with buffer solution and adjusted to pH 9.4 with one drop of  $M Na_2CO_3$ . The tissue homogenates were diluted so that readings of approximately 50 were obtained with the Evelyn colorimeter. After adding the phenol reagent, the solution was placed in the refrigerator for 15 minutes to accelerate the formation of a crystalline material which appears to be barbituric acid. The proteins and the crystalline precipitate were thrown out by centrifuging for 5 minutes at low speeds, and aliquots of the supernatant were easily pipetted. This procedure was substituted for filtration and more reliable results were obtained, particularly for the plasma. It was found that maximum color development could be attained by heating for 15 minutes at 37°. Straight-line relationships between enzyme content and optical

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density could be obtained over a wide range. Nitrogen was determined by the micro-Kjeldahl method, using copper and selenium as catalysts.

### RESULTS

*Plasma.* The activity of plasma alkaline phosphatase is influenced by the quality and quantity of the diet ingested by the rat (3). It becomes important, therefore, to

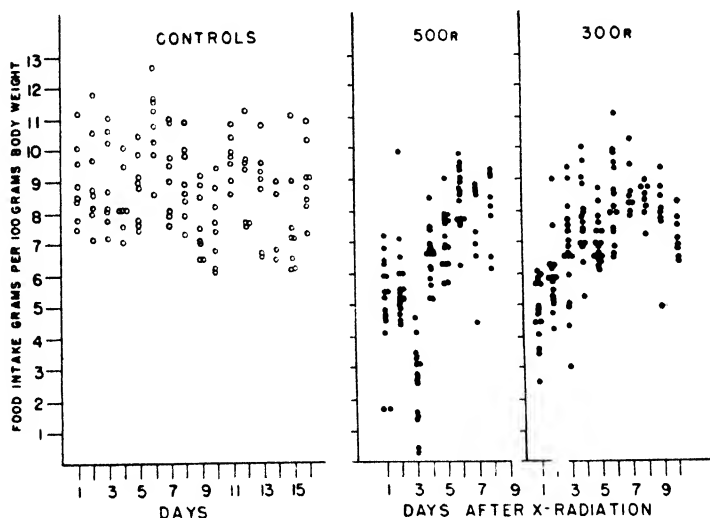


Fig. 1. DAILY FOOD INTAKE of control and irradiated rats.

TABLE 1. ALKALINE PHOSPHATASE UNITS PER 100 ML. PLASMA OF CONTROL PAIR-FED AND IRRADIATED (500 AND 600 R) RATS

DAYS	CONTROL	X-RAY
1	$42 \pm 1.72$ (23)	$52 \pm 1.44$ (8) <sup>1</sup>
2	$37 \pm 2.10$ (8)	$38 \pm 2.43$ (8)
3	$38 \pm 1.83$ (23)	$25 \pm .56$ (24) <sup>1</sup>
4	$35 \pm 2.24$ (16)	$20 \pm 1.78$ (15) <sup>1</sup>
5	$43 \pm 3.98$ (8)	$26 \pm 1.04$ (8) <sup>1</sup>
7	$30 \pm 2.58$ (16)	$26 \pm 1.97$ (15) <sup>1</sup>
10	$31 \pm 3.17$ (8)	$20 \pm 1.50$ (8)
21	$49 \pm 4.16$ (8)	$37 \pm 1.66$ (8)

Figures in parentheses represent number of animals.

<sup>1</sup> Values for *P* are less than .01.

determine the effect of irradiation on food intake. Irradiated 60-day-old male animals were placed in individual cages and the daily food (Gaines Dog Meal) intake was measured. Following exposure to 300 r (fig. 1) the food intake is decreased to its lowest level on the 1st day; after larger doses (500 and 600 r) the food intake is minimal on the 3rd day. Large variations in food intake are seen for individual control and irradiated animals on a given day. The changes in body weights of control

pair-fed animals are the same as those observed for the 500-r rats, indicating the x-radiation is not responsible for a generalized excessive tissue breakdown.

Statistical data for the alkaline phosphatase activities of the plasmas of pair-fed controls and irradiated (500- and 600-r) animals are shown in table 1. The range of values for the pair-fed animals varies between approximately 30 and 60 units with

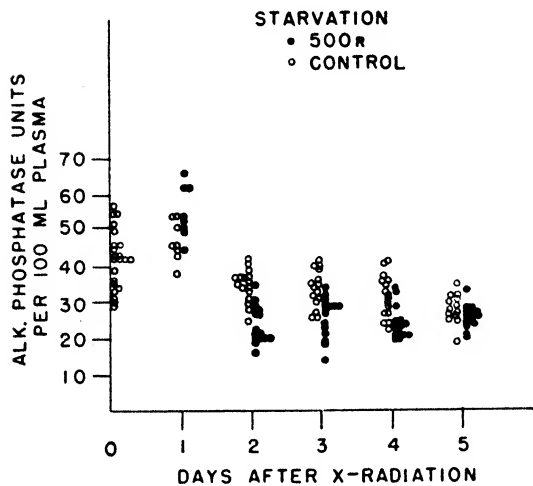


Fig. 2. ALKALINE PHOSPHATASE activity of plasma of starved controls and irradiated rats.

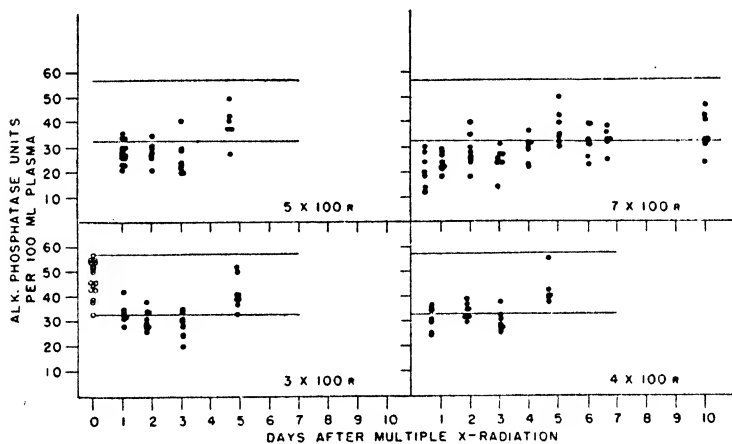


Fig. 3. PLASMA ALKALINE PHOSPHATASE activity of rats after daily multiple x-radiation. *Parallel lines* represent minimum and maximum values for control animals.

an average value of 42 units. After irradiation, the enzyme activity is increased on the 1st day, returns to the control range on the 2nd day, and is depressed during the following 5 days; the values for the pair-fed controls remain unchanged during this period. The phosphatase activity returns to the normal range on the 10th post-radiation day. At lower doses (300 r) a slight decrease in enzyme activity is observed only on the 3rd day after exposure.

Since food may influence the phosphatase content (3), the plasma alkaline phosphatase of fasted controls and irradiated rats was determined (fig. 2). After the 1st day the values for the irradiated animals are somewhat elevated. On the 2nd, 3rd and 4th days the values for the irradiated rats are significantly lower than their respective controls ( $P < .01$ ). The phosphatase activities of the starved controls and irradiated rats are the same on the 5th day. It is interesting that the phosphatase activity is not decreased below 15 units after irradiation.

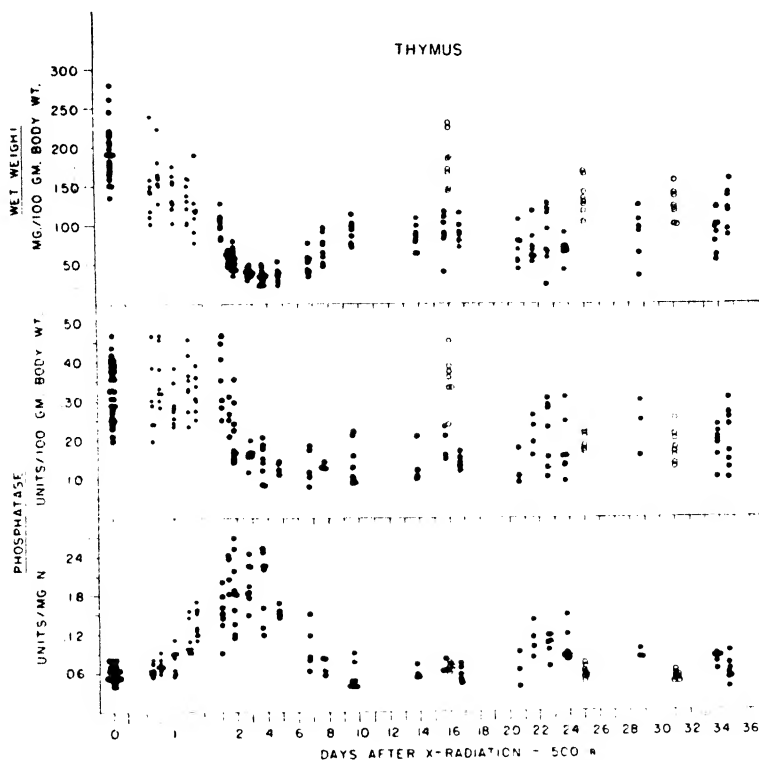


Fig. 4. EFFECT OF IRRADIATION on weight and activity and content of alkaline phosphatase of the thymus. Open circles represent values for the respective control animals.

The effect of daily small doses of x-ray on plasma phosphatase activity is shown in figure 3. These animals were not pair-fed but were deprived of food the night before they were sacrificed. It has been shown that the activity of phosphatase in the pair-fed and *ad libitum*-fed control animals is the same. Changes in activity are judged by deviations from the control range. A slight decrease in activity is observed on the 3rd day after the termination of radiation with 3 or 4 daily doses of 100 r. After higher doses ( $5 \times 100$  r and  $7 \times 100$  r) the values are markedly decreased during the first 4 days of the post-radiation period. Daily exposure to 50 r for 10 successive days causes small decreases in enzyme activity on the first 3 post-radiation days. The exposure to multiple small doses of x-ray does not depress the phosphatase activity

for so long a period or so markedly as a comparable single dose. This could be seen by comparing the effects produced after exposure to 500 r,  $5 \times 100$  r and  $10 \times 50$  r.

*Tissues.* Data are presented in figures 4 to 6 for the alkaline phosphatase activity and content and for the changes in weights of the thymus, spleen and liver after x-radiation with a dose of 500 r. Results obtained after irradiation at 300 and 600 r are similar to those shown after 500 r, and are therefore not presented. In all cases

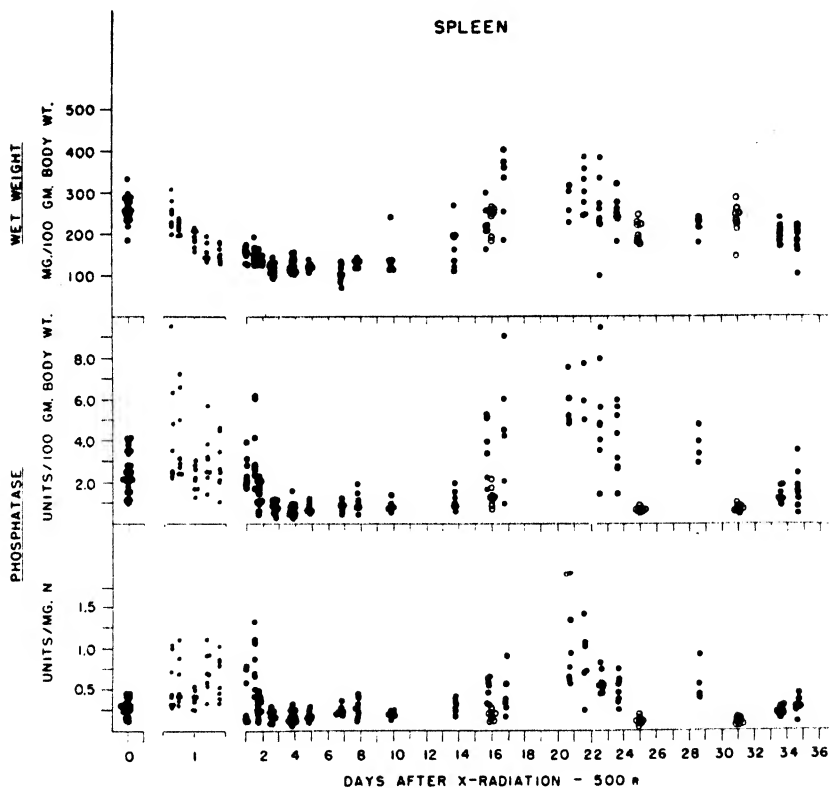


Fig. 5. EFFECT OF IRRADIATION ON weight and activity and content of alkaline phosphatase of the spleen. Open circles represent values for the respective control animals.

data are available for pair-fed control rats during the first 10 days; these are omitted since they do not differ from the control values.

*Thymus.* The thymus weights are markedly decreased immediately after irradiation and remain lower than those of the controls during the first 30 days. The enzyme activity ( $\sigma$ /mg. N) is elevated after the 12th hour following irradiation, reaches its maximum during the 2nd day and remains elevated during the next few days. This increased activity is associated with a decrease in the thymocyte content which results in a proportionately greater amount of stroma. The values are within the normal range at the end of the 1st week despite the marked involution of the thymus.

The increased values during the 4th week cannot be explained. The enzyme content per 100 gm. body weight is dependent on the relationship between activity and thymus weight and remains definitely depressed for at least 3 weeks.

*Spleen.* During the first 36 hours after irradiation, about one-half the values for phosphatase activities and contents are elevated, and at the same time the spleen is undergoing involution. After the 2nd post-radiation week, the spleens are enlarged and have a firm, rubber-like consistency with gross evidence of multiple small in-

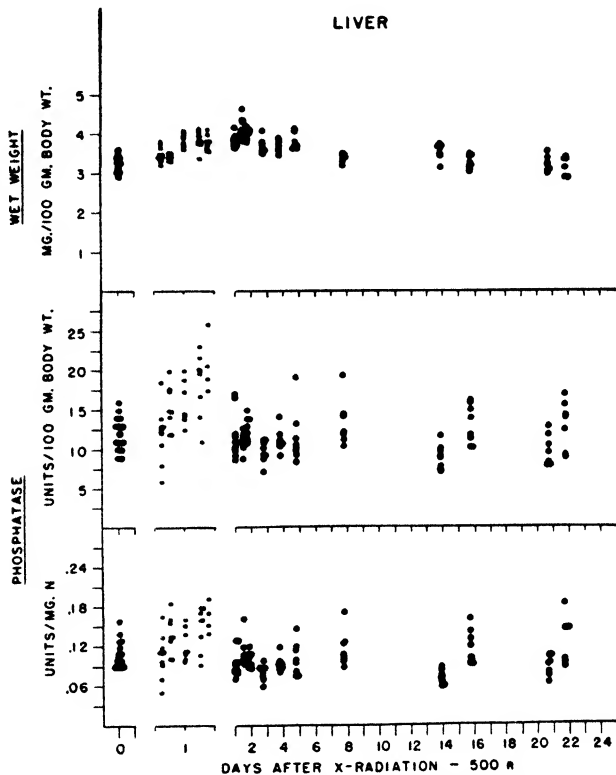


Fig. 6. EFFECT OF IRRADIATION on weight and activity and content of the alkaline phosphatase of the liver.

farcts. At this time the phosphatase activities and contents are increased. All values tend to return to the control range after 30 days.

*Liver.* A slight decrease in phosphatase activity is observed on the 2nd, 3rd and 4th post-radiation days. The livers are hypertrophied 12 hours after irradiation and remain enlarged for at least 4 days. This hypertrophy is not due to changes in the lipide and the total solids since the values for these constituents are the same as those found in the livers of pair-fed controls. Histologic examination shows a hypertrophy of the liver cells during this period.

**Kidney.** The alkaline phosphatase activity of this organ is not affected by radiation.

#### COMMENT

The plasma alkaline phosphatase activity is appreciably decreased within a few days after irradiation with non-lethal doses of x-ray. Similar decreases in this enzyme's activity are observed under the following conditions: fasting (3, 4); after hypophysectomy and injection of adrenocorticotrophic hormone (5); after administration of adrenal cortical extract (6); and following total body neutron irradiation (7). No evidence is available from the literature to indicate the mechanism responsible for the decreased plasma enzyme activity. In the present experiments no correlation could be obtained between the changes seen in the plasma and the thymus, spleen, liver and kidney.

#### SUMMARY

The activity of plasma alkaline phosphatase of rats was determined after single and multiple doses of x-rays at varying periods after total body irradiation. Relatively small decreases in phosphatase activity were found after exposure to 300 r. More marked and prolonged decreases were obtained after 500- and 600-r doses in both fed and fasted animals. Multiple daily radiation with small doses also causes a decrease in plasma enzyme activity. The alkaline phosphatase activity of the thymus is markedly increased during the first 2 days following irradiation. Small increases are observed in the spleen. Practically no change in enzyme activity is seen in the livers and kidneys of irradiated rats. The changes in the weights of the thymus, spleen and liver after irradiation are presented.

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# FORMATION OF GLUCOSE BY THE KIDNEY<sup>1</sup>

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CONSIDERABLE work has accumulated in recent years which indicates that the kidneys may be a source of blood sugar. Bergman and Drury (1) found in eviscerated rabbits that nephrectomy increases the glucose need as measured by the amount of glucose that must be injected to maintain the blood sugar at a constant normal level. In eviscerated dogs Reinecke and Hauser (2) made simultaneous analyses of arterial and venous renal blood and found higher glucose concentrations in the venous, than in the arterial blood. Cohn and Kolinsky (3) working with hepatectomized dogs found the decline in blood sugar was more rapid if the dogs had the kidneys removed than if the kidneys were left intact. Russell and Wilhelmi (4) have found glucose production by kidney slices *in vitro*.

The question of new formation of glucose by the kidney is an important one. Workers in the past have assumed that when the liver and intestines were removed, all sources of blood glucose were eliminated and hence the glucose utilization of the other tissues could be determined by measuring the rate at which glucose had to be administered to such a preparation in order to keep a constant blood sugar level. Estimates of tissue utilization made in this way varied between 75 and 250 mg/kg/hour. Should these values be increased since the workers had not included any contribution of glucose by the kidneys in their estimates? Another question requiring investigation is whether glucose utilization is increased by nephrectomy.

A third question that is of interest to workers in the field of intermediary metabolism is concerned with the nature of the substrates the kidney uses to make this glucose. Does the kidney make this glucose from protein and other non-carbohydrate sources, or does it use carbohydrate derivatives such as lactic and pyruvic acids? In the latter case little if any of such glucose would be new glucose from a balance point of view but rather glucose that had been reconstituted from fragments of former glucose molecules.

The use of radioactive glucose labeled with carbon-14 should be of value in solving these problems. By injecting glucose of known specific activity into an

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eviscerated animal and then sampling the blood glucose thereafter and determining its specific activity we can tell whether the glucose has been diluted by unlabeled glucose from some body source. By determining the production of radioactive  $\text{CO}_2$  by the animal we can tell the rate at which the glucose is oxidized.

In attacking this problem we used two methods. In the first we eviscerated rabbits, thus eliminating the liver and intestinal tract—the evident sources of new sugar. A dose of radioactive glucose was then injected, and after allowing time for mixing in the body, a sample of blood was taken. On this we determined the blood sugar concentration and the radioactivity of the  $\text{CO}_2$  produced by the action of fermentation on it. The animal was then kept for 3 or 4 hours during which time the blood sugar level dropped to a very low level. A sample of blood was then taken and the estimations of blood sugar and specific activity of  $\text{CO}_2$  produced by fermentation were repeated. It is obvious that if the specific activity of the  $\text{CO}_2$  dropped with time, that some other source of glucose must have diluted the glucose present in the body at the time of taking of the first sample. This foregoing procedure was then carried out with another animal—treated in every way the same except that in addition the kidneys were removed.

In the other type of experiment one series of animals was eviscerated (with kidneys left intact) and the blood sugar level was maintained at a constant normal level by the injection of glucose of a known radioactivity. The activity of the body glucose was first brought up to a desired level by a priming dose of potent glucose. A glucose solution was then continuously infused intravenously at a rate adequate to maintain a constant normal blood sugar level. This solution was made up to have the same radioactivity as the activity of the blood glucose resulting from the priming dose as calculated from the blood sugar level and considering the glucose space as 25 per cent of the body weight (5). To maintain a constant blood sugar, we made frequent blood sugar determinations, and kept adjusting the infusion rate accordingly. The respired  $\text{CO}_2$  was collected in portions of one hour each and the radioactivity of each was determined. This procedure was then repeated on another series subjected to bilateral nephrectomy in addition to the usual evisceration and then followed as in the previous animal. Blood samples were taken and the specific activity of the glucose of the plasmas was determined and compared with that of the injected glucose. In addition the total activity of the plasma (other than that of  $\text{CO}_2$ ) was determined by the combustion method.

#### METHODS

Evisceration of the rabbits was carried out by the 2-stage method previously described (6).

*Preparation of  $\text{C}^{14}$ -Labeled Glucose.* The radioactive glucose was uniformly labeled and was obtained by biosynthesis (7).

*Measurement of  $\text{C}^{14}$  Radioactivity.* All radioactive determinations were carried out on  $\text{BaCO}_3$  (8) obtained by either fermentation or dry combustion. In all cases the samples were of infinite thickness.

*Plasma Glucose Determination.* The plasma glucose determination was carried

out by a combination of micro fermentation with Fleischmann's Baker's yeast<sup>2</sup> as described by Reinecke (9) and the ferricyanide-ceric-sulfate macro procedure of Miller and Van Slyke (10). The plasma was deproteinized with cadmium hydroxide and the excess cadmium removed with barium carbonate. The difference between the glucose equivalent of the non-fermentable reducing substance and the conventional plasma sugar value was taken as the glucose equivalent of the fermentable reducing substance in the blood.

*Plasma Radioactivity.* For the determination of the total radioactivity (other than that of  $\text{CO}_2$ ) per unit volume of plasma, one ml. of plasma was mixed with one ml. of 0.5 M phosphate buffer. This mixture was frozen, lyophilized and weighed. The  $\text{BaC}^{14}\text{O}_3$  obtained from dry combustion was measured for radioactivity.

*Plasma Glucose Specific Activity.* The specific activity of the plasma glucose was obtained from the  $\text{CO}_2$  produced by the fermentation with Fleischmann's Baker's yeast of one ml. of plasma and 60 mg. of glucose added as carrier. The  $\text{CO}_2$  was precipitated as  $\text{BaCO}_3$ .

*Collection of Expired  $\text{CO}_2$ .* The collection of the expired  $\text{CO}_2$  was carried out by means of a tracheal cannula. A rubber balloon reservoir was placed between the animal and the collection flasks. With the aid of suction and sufficient pressure to fill the balloon the expired  $\text{CO}_2$  was collected in 2 N NaOH. The carbon dioxide was precipitated as  $\text{BaCO}_3$ .

## RESULTS

With the first type of experiment there is a clear-cut difference between the animals with and without kidneys (table 1). In the animals without kidneys the specific activity of the blood sugar remains the same as long as they live; the animals with kidneys dilute the sugar that was in their bodies immediately after evisceration with an appreciable amount of unlabeled glucose.

If all processes remained constant this situation could be submitted to mathematical analysis and the rate at which the kidney adds new sugar to the body estimated. This could be derived knowing the rate at which the kidney dilutes the body glucose and the glucose disappearance rate (as measured by the rate at which glucose must be injected to maintain a constant blood sugar level). However, it is obvious that the oxidation of glucose as judged from the  $\text{CO}_2$  production, is markedly reduced as the blood sugar level falls. The production of glucose by the kidney need not be similarly affected. This type of experiment can therefore be used only as a qualitative method.

The results of the second type of experiment are given in table 2. In these animals, after the beginning priming dose the blood sugar was kept at a normal level by the injection of a glucose solution of known activity. It may be noted that the specific activities of the plasma glucose of these animals differ little from that of the injected glucose. It would seem in these cases the kidneys did not contribute enough unlabeled glucose to dilute significantly the injected labeled glucose.

<sup>2</sup> The yeast used in these studies was generously supplied by Standard Brands Inc., San Diego, California.

*Fate of Disappearing Glucose.* In table 3 are given the figures for counts per ml. as determined by combustion and the counts per ml. as determined by fermentation. It is to be noted that at the beginning of the experiment these are about equal (after making a correction for the fact that fermentation is only 80-90% complete). In the case of most of the animals counts by combustion after 3 to 8 hours become definitely greater than counts by fermentation. This suggests that as time goes on there is the possibility of an accumulation of glucose derivatives in the circulating fluids.<sup>3</sup>

TABLE 1. EVIDENCE FOR FORMATION OF GLUCOSE BY THE KIDNEYS BASED ON SPECIFIC ACTIVITY OF PLASMA GLUCOSE

RABBIT NO.	PREPARATION	TIME OF PLASMA SAMPLING	PLASMA GLUCOSE	S.A. OF PLASMA GLUCOSE <sup>1</sup>	EXPIRED CO <sub>2</sub>	
		min. <sup>2</sup>	mg/%		min. <sup>3</sup>	gm.
5	Evisceration with intact kidneys	150	80	1600		
		210	48	1320		
		240	34	1140		
		300	20	935		
12		110	90	2240	120-180	2.33
		255	44	1630	180-240	1.39
		360	26	1000	240-300	1.12
					300-360	0.81
13	Evisceration kidneys removed	85	126	10,300	90-150	2.41
		265	22	12,600	150-210	1.47
					210-270	0.78
26		150	138	2850		
		170	128	2860		
		330	42	3120		

<sup>1</sup> Specific activity is the count of total carbon/mg./min.

<sup>2</sup> Zero minutes begins at start of evisceration which takes approximately one hour. *Rabbits 12 and 13* received 241 and 284 mg. of radioactive glucose intravenously, respectively, 15 min. before start of operation. *Rabbit 5* received 75 mg. of radioactive glucose at 120 min. and *rabbit 26* received 69 mg. at 130 min.

In the intact animal these would not accumulate to any significant degree since they would be reconverted to glucose by the liver. Some of these substances may act as substrates for glucose formation by the kidney. Such substances would have essentially the same specific activity as that of the glucose they were derived from and hence the reconverted glucose would not reduce the activity of the circulating glucose. In this case there would be evidence of glucose formation by the kidney from arterio-venous differences of blood sugar as determined by chemical procedure but not by dilution of activity of circulating glucose.

<sup>3</sup> Such an effect could result from the presence in the injected glucose of impurities having radioactivity but it is most unlikely that such a high degree of contamination could have persisted during the purification of the glucose (11). The specific activity of the carbon dioxide obtained by fermentation and total combustion of the injected glucose are the same.

## DISCUSSION

Our results support the conclusion that the kidneys, at least under certain circumstances, can form glucose. The condition in addition to the evisceration that we find necessary for this is that the blood sugar be allowed to fall to hypoglycemic levels.

There is no evidence of sugar formation by the kidney if the liver is intact; the blood sugar does not fall after nephrectomy alone, and Reinecke and Hauser (2) did not find higher values for renal vein blood than arterial blood in intact animals. Their results show definitely higher renal vein blood than arterial blood only when the blood

TABLE 2. COMPARISON OF SPECIFIC ACTIVITY OF INJECTED GLUCOSE WITH THAT OF PLASMA GLUCOSE AFTER 8 HOURS CONTINUOUS INFUSION

RABBIT NO.	SPECIFIC ACTIVITY 1ST SAMPLE	SPECIFIC ACTIVITY, 2ND SAMPLE (8 HOURS LATER)	SPECIFIC ACTIVITY OF INJECTED GLUCOSE
23	2950	3540	3880
29		2540	2890
30	3330	2560	2370
39	3200	2500	2815

TABLE 3. COMPARISON OF TOTAL PLASMA RADIOACTIVITY BY COMBUSTION AND FERMENTATION<sup>1</sup>

	RABBIT NO.	TIME OF SAMPLING AFTER INJECTION OF GLUCOSE					
		0.5 - 1 Hour		3.5 Hours		8 Hours	
		Ferment.	Combust.	Ferment.	Combust.	Ferment.	Combust.
Kidneys intact	30	1270	1480			920	1500
	29	1610	1450			1140	1930
	23	1470	1450			1560	1930
	14					1400	3340
Nephrectomized	19			1090	2720		
	18					4270	7130

<sup>1</sup> Expressed as counts/ml/min.

sugar levels were hypoglycemic. Roberts and Samuels (12) found no sugar differences between renal vein and arterial blood in intact rats. In all reported work showing positive production of glucose by the kidney the liver has been removed and the blood sugar has been allowed to fall. This might mean that the hypoglycemic syndrome may activate the sugar-making function of the kidney. On the other hand as a result of the hypoglycemia there may be liberated into the blood stream a substrate that the kidney can change to glucose; lactic acid comes to mind in this connection.

With the kidneys removed but with the blood sugar level maintained at a normal level we obtained little if any evidence of sugar formation by the kidney. This indicates that the kidneys in our experiments at times may have been making sugar out of substrates derived from the circulating glucose.

Insofar as this work bears on the problem of intermediary metabolism and carbohydrate balance there is no basis for increasing the former estimate of glucose utilization by the tissues during fasting. It appears that in glucose-balance studies carried out after removal of the liver and when the blood sugar has been maintained at normal levels that if the kidneys formed additional glucose, they formed it from fragments of glucose molecules. This would be a cycle mechanism and would not mean any overall increase in glucose utilization by the tissues. Roberts and Samuels (12) report evidence of sugar formation by the kidney by the arterio-venous difference method. The blood samples were taken 6 hours after evisceration and no sugar was given. However, some of their animals were still above the hypoglycemic level. This can be reconciled with our findings if the kidneys use as a substrate for gluconeogenesis glucose breakdown products in addition to compounds which may come from other sources. The former seem to increase progressively in concentration after evisceration and it is to be expected that more and more glucose is formed by the kidney from this source as time goes on.

There remains to consider the results of Bergman and Drury (1) in which the blood sugar was maintained at normal levels and it was found that when the kidneys were removed the eviscerate rabbit had a higher glucose disappearance rate. Similar findings have been obtained by Ingle and Nezamis (13) in the rat. In the light of our findings this could be explained by an increase in incomplete breakdown of glucose as a result of kidney removal since the oxidation rate of glucose in the eviscerate animal is not materially reduced by removal of the kidneys (14). On the other hand these findings may be explained by the formation of glucose by the kidney from breakdown products of glucose. Very likely it is a combination of these two factors.

#### SUMMARY

The production of glucose by the kidney was studied in eviscerate rabbits, using radioactive glucose as a tracer. The specific activity of the circulating glucose was followed as an indicator of any addition of new glucose from a body source. After one injection of marked glucose and then allowing the animals to become hypoglycemic, the specific activity of the circulating glucose would be reduced if the kidneys were left intact. The activity was not changed if the kidneys were removed. This indicates that the kidney makes new glucose under these conditions.

When the blood sugar was maintained for 8 hours at a normal level by injection of glucose solution of known radioactivity, the specific activity of the terminal plasma glucose was found to be similar to that of the injected glucose, indicating that no appreciable amount of new glucose was added by the kidneys. Evidence is presented which supports the view that the kidney in these circumstances may form glucose from fragments of circulating glucose.

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# SEX DIFFERENCE IN PROTEINURIA IN THE RAT<sup>1</sup>

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THE rat normally excretes a measurable amount of protein in its urine (1, 2). It has been established that the rate of protein excretion in the adult male rat is many times greater than in the adult female and that this difference in proteinuria becomes manifest at, or shortly after, puberty (50-60 days of age) (1, 3, 4). Wicks (5) has shown that a similar sex difference in proteinuria exists in the mouse.

The nature of the excreted protein and its source in the genito-urinary tract have not been settled with any degree of certainty. Shih (3) has maintained "that the high rate of protein excretion in the male is due to addition of extra-renal protein." Bell (4) and Addis (1) attributed the additional protein found in the male rat to contamination of the urine by prostatic secretions. Wicks, however, found the protein content of bladder urine of mice drawn immediately after kill to be as high as that of voided urine, and concluded that the protein found in the urine is of renal origin. He has identified the protein excreted by the mouse as a nucleoprotein while Parfentjiv and Perlzweig (6) believed it to be a chondromucoid substance.

In this report, the sex difference in proteinuria of male and female rats is confirmed and data will be presented which indicate that at least the major portion of the excreted protein of male and female rats takes its origin in the kidney. The effect of castration and the administration of testosterone propionate<sup>3</sup> on proteinuria will be described.

## MATERIALS AND METHODS

*Urine Collections.* Animals were taken off their usual stock diet at 3 P.M. and put in individual metabolism cages placed over funnels. The top of the funnel was covered with a wire mesh to avoid fecal contamination of the urine. At 8 A.M. the following morning the collection period was terminated. During this 17-hour overnight period the animals subsisted on a 15 per cent solution of glucose in 0.4 per cent sodium chloride containing 0.5 per cent of a solution of B vitamins (Betaplexin). Their 17-hour overnight urine volumes averaged 45 ml.

*Protein Determinations.* Urinary proteins were determined by a modification of

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Kingsley's biuret method (7) on the urines excreted by the individual rats during the 17-hour overnight collection period.

*Animals.* Male and female albino rats of the Slonaker-Addis strain were used throughout these studies.

#### PROCEDURES AND RESULTS

*Change in Proteinuria with Age.* Three groups of animals were used in this experiment. *Group A* consisted of 24 males, *group B*, 24 females, and *group C*, 12 males that were castrated at 23 days of age. Starting at 30 days of age, urines were collected

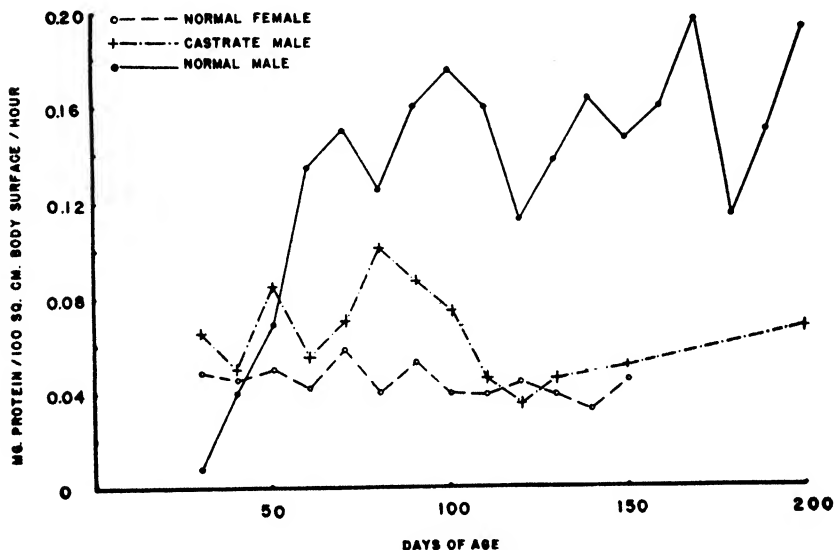


Fig. 1. EFFECT OF AGE ON rate of protein excretion in urine of albino rats.

during the 17-hour overnight period and analyzed for protein. The overnight urine collections were repeated at 10-day intervals for the duration of the experiment.

The data are illustrated in figure 1. Each point represented on the graph is the average of protein determinations performed on the urines of the individual rats in the group indicated. The results are expressed as mg. protein/hr/100 cm.<sup>2</sup> of body surface in order to allow comparison of animals with different body weights and kidney mass.<sup>4</sup> It can be seen that the rate of protein excretion in the urine of normal male rats begins to exceed that of normal female rats at about 50 days of age. The increase is rapid, and the male-female difference persists for at least 200 days at which time the experiment was terminated.

In the castrate male, the rise in proteinuria is sharply reduced, as compared to

<sup>4</sup> Since protein excretion in the urine may be a function of total kidney size, it was thought best to express proteinuria in terms of some unit of kidney mass. In the rat, kidney mass is most closely correlated with body surface area (8), hence our results are expressed as mg./hour/100 cm.<sup>2</sup> of body surface.



that in the normal male, but protein excretion does not fall to the normal female level until about 110 days of age. These data are in close agreement with those presented by Addis (1).

*Source of Urinary Protein.* Two groups of animals were used in this experiment. *Group A* consisted of 24 males weighing 300 to 350 gm. and *group B* consisted of 24 females of 175 to 200-gm. body weight. The urines from all animals were collected during a 17-hour overnight period and analyzed for their protein content. The average values are shown in table 1, under the column entitled 'Voided.' The following morning, each animal received 5 cc. of physiological saline solution subcutaneously and a solution containing 4 mg. of sodium pentobarbital (Nembutal)/100 gm. of body weight in normal saline intraperitoneally. Their bladders were emptied at once by allowing the animals to sniff ether, and simultaneously exerting pressure over the bladder area. The animals became unconscious within 5 to 10 minutes and at the end of 60 minutes were still adequately anesthetized. At this time, the urinary bladder was carefully exposed through an abdominal incision and was in all instances tightly distended with clear urine. The base of the bladder was clamped with a curved mosquito forceps and the urine aspirated through a 26-gauge needle inserted into the bladder through an avascular portion of its wall. Care was taken to aspirate the urine precisely 60 minutes after the start of the urine collection period.

The bladder urine was then analyzed for its protein content. It was necessary to pool the individual urines in each sex group in order to obtain enough material on which to do accurate protein determinations. These results are indicated in table 1. The protein content of the voided urine reveals the same sex difference as is demonstrated in figure 1. The protein content of the bladder urine, presumably free from lower urinary tract contamination, likewise is five times greater in the male than in the female. The difference in protein content between the voided and bladder urine of the male is not great, and since they were collected on consecutive days, is consistent with the normal degree of day-to-day variation in proteinuria that is seen in this species (fig. 1). The difference in protein content of the voided and bladder urine of the female is slightly greater. At the low level of proteinuria found in the female, differences of this degree lose much of their significance. The data indicate that the protein found in male and female voided urine comes for the most part directly from the kidney, although the urinary bladder is not entirely eliminated as a minor source of some excreted protein. The question of whether or not the accessory sex glands contribute to the increased proteinuria found in the male would appear to be largely academic with the finding of a similar sex difference in proteinuria in the bladder urine.

*Effect of Testosterone Propionate Injections on Proteinuria of Castrate, Adult, Male and Female Rats.* Twenty-four male and 24 female rats were employed in this experiment. Urines were collected from all rats during the 17-hour overnight period and analyzed individually for their protein content. This was done on the 1st and 4th days of the experiment. Each point represented in figure 2 is the average of the urinary protein concentration of the 24 animals in the respective sex group. The first two urinary protein determinations represent the pre-operative control levels of proteinuria of each group of animals. All rats were castrated on the 5th and 6th

days of the experiment and overnight urine collections for protein determinations were run at 7- to 10-day intervals throughout the course of the experiment. It is seen in figure 2 that the protein content of male urine fell precipitously after castration and then leveled off at a value approximately half way between its former figure and the female value. The protein content of female voided urine was essentially unchanged by castration. When it became apparent that the degree of proteinuria in the castrate males and females had reached a constant basal level, 2.0 mg. of testosterone propionate was injected subcutaneously daily in all animals.

After a latent period of 6 days, in the male, the protein content of the urine began to mount sharply. Castrate males receiving 2.0 mg. of testosterone propionate daily finally reached a degree of proteinuria of the same magnitude as the normal intact male receiving no therapy. After a latent period of 11 days, castrate females receiving testosterone also developed a sharp increase in proteinuria reaching levels equal to those of the untreated castrate males. This represents a twofold increase in protein excretion as a result of testosterone administration. The question of contamination of the voided urine by lower urinary tract secretion need not be seriously

TABLE 1. PROTEIN CONTENT OF VOIDED AND BLADDER URINE IN NORMAL MALE AND FEMALE RATS

SEX	NO. OF ANIMALS	PROTEIN CONTENT OF URINE	
		Voided	Bladder
		<i>mg/hr/100 c m.<sup>2</sup> body surface</i>	
Male	24	0.12	0.10
Female	24	0.05	0.02

considered in the female rat. The increased protein excretion due to testosterone administration must be directly of renal origin.

#### DISCUSSION

When the male rat is castrated before puberty, its protein excretion diminishes after a time to the normal female level (fig. 1). When, however, the adult male rat is castrated, the level of protein in its urine remains half way between the normal male and female levels (fig. 2). The explanation for this difference in the behavior of the castrate rat depending upon its age at castration is not at all clear. It is possible that when the male kidney has been under the influence of the functioning testes for some extended period of time, irreversible changes in its structure occur leading to increased proteinuria. Thus, an increase in protein excretion over and above that found in the female persists following orchidectomy in the adult rat. The kidney of the pre-puberty rat on the other hand has been affected little, if at all, by androgenic substances and hence is capable of very low levels of protein excretion.

The male-female difference in proteinuria in the rat would seem at first hand to be the result of increased androgen production in the male. This hypothesis is strengthened by the observation that orchidectomy reduces the rate of protein excretion. The demonstration that the sex difference in proteinuria persists in bladder urine, uncontaminated by lower urinary tract secretions, indicates that if androgens

are responsible for the increased protein excreted by the male, their site of action is ultimately on the kidney.

Since the original observation by Korenchevsky and Dennison (9) that androgens restore the kidney size of castrate male rats, much has been written on the effects of various androgen preparations, especially testosterone propionate, upon renal morphology and function (10-12). There is no doubt that striking changes in the size and structure of the kidney may be produced by androgen administration, and testosterone propionate seems most active in this regard. Insofar as we have been able to determine, no mention has been made in the literature on the effect of this class of compounds on protein excretion by the kidney.

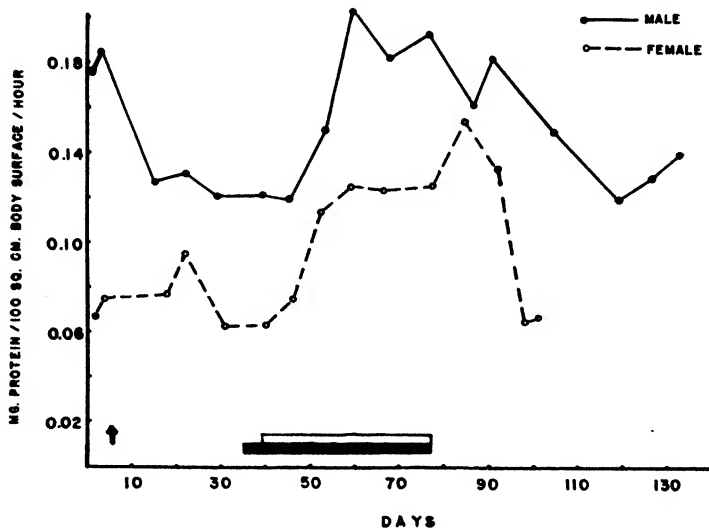


Fig. 2. EFFECT OF TESTOSTERONE PROPIONATE administration on rate of protein excretion in urine of castrate, adult, male and female albino rats. Castration was performed as indicated by the arrow. Solid black indicates duration of testosterone propionate administration to female rats; while block indicates duration of testosterone propionate administration to male rats.

It is clear that testosterone propionate in the dosage used in these experiments results in increased protein excretion in both male and female castrate rats. The fact that testosterone increases the proteinuria of castrate females indicates that the site of action of this compound is on the kidney and not on the accessory sex glands.

While these results indicate that the sex difference in proteinuria is a result of action on the kidney by androgen produced in the male testis, the exact mechanism of this interaction is not clear. Selye (10) has shown that testosterone produces hypertrophy of the epithelium lining the parietal lamina of Bowman's capsule, while Selye (10), Korenchevsky and Ross (13), and others have demonstrated hypertrophy of the epithelium of the proximal and distal convoluted tubules. Whether these histological changes are sufficient to alter the glomerular permeability to protein or to diminish the ability of the tubule to reabsorb the protein which may normally be present in the glomerular filtrate, is not at present known.

## SUMMARY

Adult male rats of the Slonaker-Addis strain excrete four to five times more protein in their urine than adult females. The male-female difference in protein excretion found in voided urine is similarly found in urine taken directly from the urinary bladder. It is concluded that the increased protein found in the urine of male rats is of renal origin.

Castration of adult male rats results in a sharp fall in proteinuria. Castration of adult female rats does not affect the quantity of protein excreted in their urine. Treatment with subcutaneous injections of 2.0 mg. of testosterone propionate daily greatly increases the rate of protein excretion in both male and female castrate rats.

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# BIOCHEMICAL, CELLULAR, AND BACTERIOLOGIC CHANGES IN THORACIC DUCT LYMPH OF DOGS EXPOSED TO TOTAL BODY IRRADIATION<sup>1</sup>

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**B**LOOD and fixed tissues of irradiated animals have been extensively studied, but the extra-vascular fluids have received the attention of relatively few investigators (1,2). A summary of the literature by Drinker and Yoffey (3) shows the close similarity of the lymph and tissue fluids. It was felt, therefore, that it would be valuable to study the changes in lymph composition following x-radiation and to compare them with the changes which have been found to occur in blood following x-radiation.

## METHODS AND MATERIALS

Young male dogs weighing from 25 to 45 pounds were used. The animals received Purina Laboratory Chow and water *ad libitum* for 2 weeks prior to use. All dogs were fasted for 12 hours prior to surgery.

Veterinary sodium pentobarbital (Nembutal) (1 cc/5 lb. body weight) was the anesthesia used. The dogs were kept anesthetized throughout the experiments by the use of additional Nembutal when indicated. The thoracic duct was isolated using established techniques (4,5) and was cannulated with siliconed glass tubing. When indicated, heparin on a fine wire loop was placed in the neck of the cannula to decrease the clotting tendency of the lymph. Solutions of distilled water containing either 5 per cent glucose or normal saline were given slowly into the femoral vein in amounts approximately equal to the amount of lymph lost from the fistula.

A heavy duty x-ray generator was used to irradiate the animals. The physical factors of the generator are as follows: 2000 K.V.P.; 1.5 M.A.; radial beam; no added filters; 15 r per minute in air at 2 meters distance (6). The animals received 500 r total body irradiation delivered to one lateral aspect.

Our observations on the thoracic duct lymph of normal and x-radiated dogs were divided into 3 chief categories: *a*) biochemical, *b*) cellular, and *c*) bacteriologic. The biochemical determination of total protein was made by the micro-Kjeldahl method (7); the protein-free filtrate, non-protein nitrogen and glucose determinations were made by the Folin-Wu procedures (7); creatinine and uric acid by the method of Folin (7). Because of the low uric acid levels in dogs, a one-to-four dilution was made

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<sup>1</sup> The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

of the uric acid standard, and 10 cc. of the filtrate were used. Chloride determinations were made by the method of Sendroy as modified by Van Slyke and Hiller (7).

Complete blood cell counts and clotting times were made on the lymph samples. Lymph cell counts were done in duplicate in the same manner as blood counts. Giemsa stain was used in the differential smears.

Bacterial cultures of blood and lymph were taken in many of the experiments. The cultures were made by flaming the tip of the cannula, inserting a long 18-gauge needle into the cannula, and withdrawing 2 cc. of lymph into a sterile syringe. The lymph was then inoculated into 25 cc. of beef-heart-infusion broth. Cultures were also taken on eosin-methylene-blue agar plates. Incubation time was 72 hours.

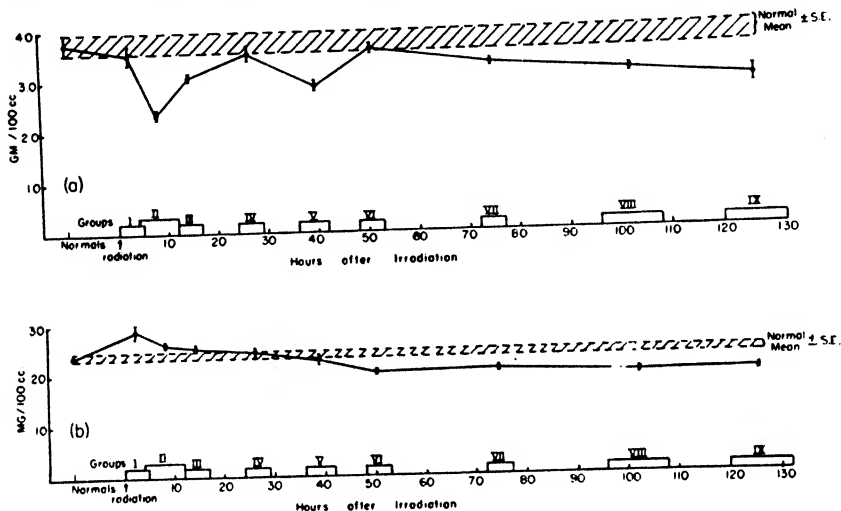


Fig. 1. TOTAL PROTEIN (a) and NPN (b) values of thoracic duct lymph in normal and irradiated dogs. Mean values and standard errors are plotted for each experimental group (see table 1a and b).

Ten-cc. lymph samples were collected every 1 to 2 hours in small bottles containing dried lithium oxalate (1 cc. of a 2% solution). Oxalated blood samples were taken during lymph collection periods in some of the experiments. After samples for cell counts had been withdrawn, the blood was centrifuged and the plasma removed for biochemical analysis.

The first post-operative lymph samples from each of 22 non-irradiated dogs served as the control specimens. Thirty-three dogs were divided into 8 groups, of 2 to 6 dogs each, representing various time intervals from 4 to 132 hours post-irradiation. Seven dogs from which control samples were taken were later irradiated and comprise group I of the experimental series.

#### RESULTS

In thoracic duct lymph of the 22 normal dogs we obtained values for total protein varying from 2.45 to 6.63 gm. per cent with an average of 3.75 gm. per cent.

This is in close agreement with published results (3). Following irradiation, the trend of the total protein values is downward (fig. 1*a*, table 1*a*). Fluctuations occur within the first 50 hours, but after this period, the data become more stable.

NPN values in the lymph of normal dogs vary from 16.9 to 30.7 mg. per cent with an average of 23.8 mg. per cent. There is an increase to approximately 21 per cent above normal in the NPN levels within the first 5 hours after irradiation. After this time there is a gradual return to normal by the 30th hour, after which they con-

TABLE 1. TOTAL PROTEIN AND NPN VALUES OF THORACIC DUCT LYMPH AND PLASMA IN NORMAL AND IRRADIATED DOGS

EXPTL. GROUP	FLUID	NO. OF DOGS	TOTAL PROTEIN (a)			NPN (b)		
			No. of determinations	Range	Mean	No. of determinations	Range	Mean
				gm/100 cc.	gm/100 cc.		mg/100 cc.	mg/100 cc.
<i>Controls</i>	L	22	22	2.45-6.63	3.75	22	16.9-30.7	23.8
	P	3	9	3.45-5.32	4.42	9	18.7-27.2	23.6
<i>I, 0-5 hr.</i>	L	7	37	1.80-7.59	3.53	39	18.1-49.5	28.9
	P	0	0			0		
<i>II, 4-12 hr.</i>	L	2	10	1.96-2.80	2.36	10	23.7-28.1	26.0
	P	2	6	3.75-4.24	4.00	6	23.7-26.4	24.8
<i>III, 12-17 hr.</i>	L	4	24	2.56-4.39	3.09	24	18.7-31.0	25.4
	P	0	0			0		
<i>IV, 24-29 hr.</i>	L	6	34	2.02-5.86	3.56	35	16.3-35.9	24.7
	P	0	0			0		
<i>V, 36-42 hr.</i>	L	3	14	2.20-3.71	2.89	14	16.8-31.0	22.9
	P	2	4	3.45-5.36	4.16	4	17.8-28.1	22.7
<i>VI, 48-53 hr.</i>	L	5	21	2.81-4.69	3.60	20	16.2-23.1	20.1
	P	0	0			0		
<i>VII, 72-77 hr.</i>	L	6	33	2.77-4.07	3.25	34	13.7-24.7	20.9
	P	0	0			0		
<i>VIII, 96-108 hr.</i>	L	3	20	2.48-3.70	3.08	20	17.9-23.7	20.1
	P	2	5	4.25-5.27	4.67	5	20.9-23.8	22.4
<i>IX, 120-132 hr.</i>	L	4	20	1.78-4.23	2.86	20	16.6-24.9	20.4
	P	4	11	2.99-5.00	4.24	11	15.8-23.7	21.1

L: lymph; P: plasma.

tinue to drop and finally reach a level which is 86 per cent of normal at the end of the 132-hour observation period (fig. 1*b*, table 1*b*).

Normal creatinine values in lymph range from 1.05 to 2.49 mg. per cent with a mean of 1.49 mg. per cent. There is a decrease to 70 per cent of the normal by 17 hours after irradiation. Very slight deviations occur thereafter, the creatinine returning to approximately 89 per cent of normal at 132 hours (fig. 2*a*, table 2*a*).

Sugar levels of thoracic duct lymph range from 88 to 168 mg. per cent with an average of 134 mg. per cent. Very slight changes are observed in the sugar levels of lymph of irradiated dogs (fig. 2*b*, table 2*b*).

Uric acid levels in normal lymph fall between 0.25 and 0.67 mg. per cent with a mean of 0.41 mg. per cent. Within the first 5 hours after irradiation and continuing

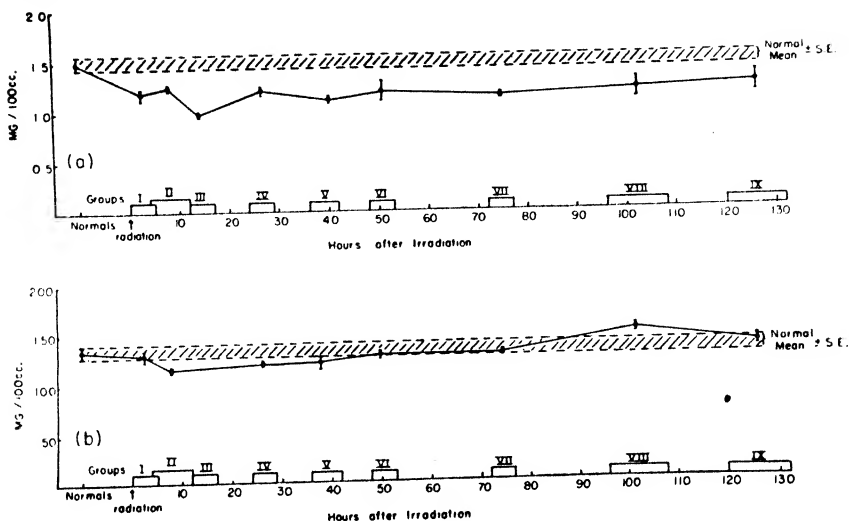


Fig. 2. CREATININE (a) and sugar (b) values in thoracic duct lymph of normal and irradiated dogs. Mean values and standard errors are plotted for each experimental group (see table 2a and b).

TABLE 2. CREATININE AND SUGAR VALUES OF THORACIC DUCT LYMPH AND PLASMA IN NORMAL AND IRRADIATED DOGS

EXPTL. GROUP	FLUID	CREATININE (a)				SUGAR (b)			
		No. of dogs	No. of determinations	Range	Mean	No. of dogs	No. of determinations	Range	Mean
				mg/100 cc.	mg/100 cc.			mg/100 cc.	mg/100 cc.
Controls	L	18	18	1.05-2.49	1.49	18	20	88-168	134
	P	3	9	1.07-1.59	1.27	3	9	107-138	125
I, 0-5 hr.	L	4	22	0.86-1.49	1.19	4	33	78-190	128
	P	0	0			0	0		
II, 4-12 hr.	L	2	10	1.07-1.54	1.25	2	10	108-118	113
	P	2	6	1.33-1.56	1.43	2	6	94-99	96
III, 12-17 hr.	L	4	24	0.73-1.55	0.99	0	0		
	P	0	0			0	0		
IV, 24-29 hr.	L	5	29	0.75-1.87	1.22	5	18	100-143	120
	P	0	0			0	0		
V, 36-42 hr.	L	3	14	1.14-1.29	1.21	2	8	98-150	121
	P	2	4	1.18-1.38	1.28	2	4	80-125	104
VI, 48-53 hr.	L	4	23	0.93-1.50	1.13	2	9	111-150	127
	P	0	0			0	0		
VII, 72-77 hr.	L	6	34	0.88-1.40	1.17	5	28	105-143	127
	P	0	0			0	0		
VIII, 96-108 hr.	L	3	20	1.07-1.40	1.22	3	20	123-187	152
	P	2	6	1.17-1.40	1.28	2	6	118-158	144
IX, 120-132 hr.	L	4	20	1.09-1.47	1.26	4	20	92-171	137
	P	4	11	1.16-1.45	1.29	4	11	96-107	122

L: lymph; P: plasma.



through the 29th hour, uric acid levels are markedly elevated to 172 per cent of normal. The levels drop to normal in the 5th observation period, decrease further by the 6th, and remain slightly below normal for the rest of the experiment (fig. 3*a*, table 3*a*).

Chloride determinations were made on only a few dogs and show essentially no change following irradiation.

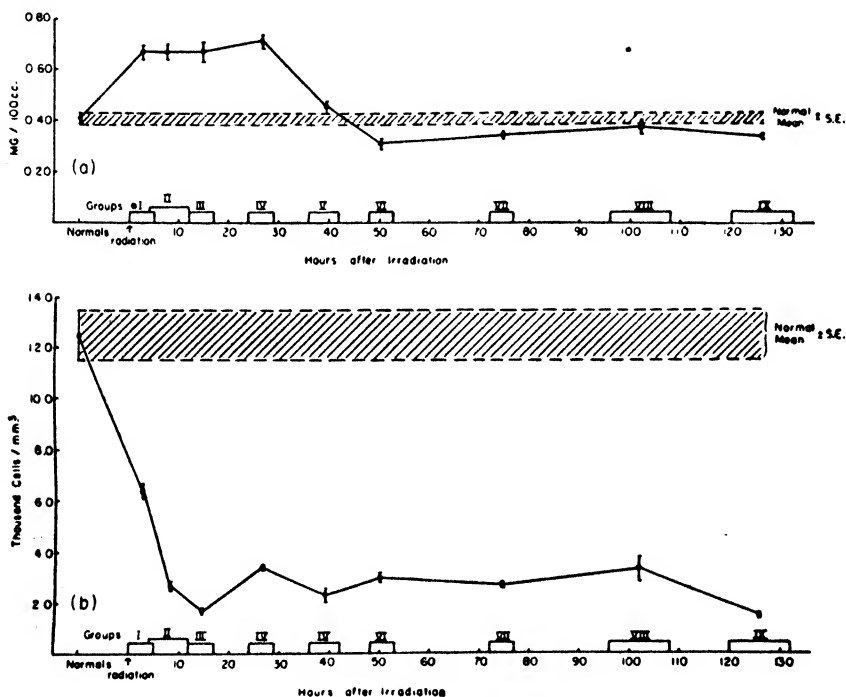


Fig. 3. URIC ACID levels (a) and total white blood cell counts (b) of thoracic duct lymph in normal and irradiated dogs. Mean values and standard errors are plotted for each experimental group (see table 3*a* and *b*).

We observed a range of 4,700 to 22,500 with a mean of 12,500 total white blood cells per cubic millimeter of lymph on normal dogs. By 18 hours after irradiation, lymphocytes decrease to 18 per cent of normal. Slight variations occur from the 24th to the 120th hours with an average depression to 23 per cent of normal. By the 132nd hour following irradiation lymphocytes drop to 13 per cent of normal (fig. 3*b*, table 3*b*). The relative cell percentages remain unaltered throughout.

The rate of lymph flow varies from 0.1 cc/minute to 1.25 cc/minute and decreases in the majority of animals during each experiment. The decrease is constant and gradual in the normal control as well as in the irradiated dogs.

In general, where blood and lymph samples are obtained simultaneously there are only slight differences in the concentrations of the biochemical substances studied.

Protein levels are higher in plasma than in lymph. Plasma uric acid levels tend to be slightly lower than in lymph and do not increase after irradiation at the time when lymph uric acid increases considerably. Blood and lymph cell counts respond similarly to irradiation.

Lymph clotting times done on samples taken at frequent intervals show a remarkably varied and inconsistent pattern which can not be correlated with any other observation.

TABLE 3. URIC ACID LEVELS AND TOTAL WHITE BLOOD CELL COUNTS IN THORACIC DUCT LYMPH AND PLASMA OR BLOOD IN NORMAL AND IRRADIATED DOGS

EXPTL. GROUP	URIC ACID (a)					TOTAL WHITE BLOOD CELLS IN LYMPH (b)				
	Fluid	No. of dogs	No. of determinations	Range	Mean	Fluid	No. of dogs	No. of determinations	Range	Mean
				mg/100 cc.	mg./100 cc.				cells/cu. mm.	cells/cu. mm.
<i>Controls</i>	L	21	21	0.25-0.67	0.41	L	17	17	4,700-19,100	12,500
	P	3	9	0.18-0.48	0.30	B	10	10	5,100-19,000	12,000
<i>I, 0-5 hr.</i>	L	6	35	0.37-1.10	0.67	L	7	43	1,700-13,000	5,300
	P	0	0			B	1	4	4,500-6,800	5,900
<i>II, 4-12 hr.</i>	L	2	10	0.46-0.86	0.65	L	2	10	1,800-4,200	2,700
	P	2	6	0.35-0.46	0.39	B	2	6	8,700-14,000	10,300
<i>III, 12-17 hr.</i>	L	3	18	0.44-0.98	0.65	L	4	24	900-3,500	1,700
	P	0	0			B	0	0		
<i>IV, 24-29 hr.</i>	L	6	35	0.35-0.98	0.71	L	6	21	1,400-8,000	3,400
	P	0	0			B	4	21	4,100-19,700	10,150
<i>V, 36-42 hr.</i>	L	3	14	0.30-0.55	0.45	L	3	14	950-4,400	2,300
	P	2	4	0.27-0.38	0.33	B	2	4	5,500-7,500	6,400
<i>VI, 48-53 hr.</i>	L	5	29	0.11-0.58	0.30	L	5	30	1,400-5,800	3,000
	P	0	0			B	0	0		
<i>VII, 72-77 hr.</i>	L	6	34	0.18-0.63	0.34	L	6	34	1,900-3,500	2,700
	P	0	0			B	0	0		
<i>VIII, 96-108 hr.</i>	L	3	20	0.15-0.50	0.37	L	3	20	400-7,900	3,300
	P	2	6	0.41-0.48	0.44	B	2	10	3,000-7,300	4,600
<i>IX, 120-132 hr.</i>	L	4	17	0.28-0.37	0.33	L	4	21	650-2,700	1,500
	P	4	10	0.26-0.34	0.30	B	4	14	850-3,100	1,500

L: lymph; P: plasma; B: blood.

#### DISCUSSION

The study of lymph presents many problems which make biochemical analyses difficult to interpret. In the first place the introduction of certain stresses such as anesthesia and surgery are necessary to expose the duct and collect the lymph. These stresses are capable of producing non-specific reactions, manifesting themselves in biochemical and cellular changes (8).

Secondly, normal healthy animals have only occasional red blood cells in thoracic duct lymph, but we noted the spontaneous and intermittent appearance of erythrocytes in several dogs, while in others the lymph was bloody throughout the experiment. The origin of the blood in lymph has not been established (3), but we believe

with Drinker (5) that it has two possible sources: parasitic infestation of the gut, and/or increased permeability of the endothelium of the liver sinusoids.

The nature of thoracic duct lymph, coming as it does from the extremities and abdominal organs, is normally subject to fluctuations in composition. In the anesthetized animal, thoracic duct lymph comes predominately from the intestines (3) and has, therefore, a fairly uniform composition.

The variables are present in both the irradiated and control dogs, but they have been kept as uniform as possible in all experiments. We feel, therefore, that the alterations observed reflect for the most part the effect of x-radiation.

We can offer no one explanation for the fall in total protein following total body x-radiation. However, the dogs may develop a negative nitrogen balance (9) which with other factors such as inactivation of intracellular enzymes (10,11), denaturation of proteins, and cellular destruction may contribute to the protein decrease observed in irradiated dogs.

Injured cells release excessive amounts of non-protein nitrogenous substances, especially nucleic acids, which are hydrolyzed to form uric acid. Lymphocytes, which are among the most radio-sensitive cells of the body, contain a high concentration of nucleic acids. The high uric acid levels observed in the lymph of irradiated dogs coincide with the period of greatest lymphocyte destruction. The post-irradiation rise in NPN levels of the lymph also coincides with the period of greatest cell destruction.

We have no adequate explanation of the observed decrease in lymph creatinine concentration.

All cultures of blood and lymph were negative. Post-irradiation bacteremia usually does not appear until the animals are moribund, or until two weeks or more after exposure. Our observations did not go beyond 132 hours.

#### SUMMARY

Biochemical, cellular, and bacteriologic studies were conducted on thoracic duct lymph and blood in normal dogs and in dogs exposed to 500 r total body x-radiation. Lymph total protein fluctuated within the first 50 hours after irradiation and then decreased at a slow, even rate. Uric acid and non-protein nitrogen levels in lymph increased within 4 hours following irradiation, but later decreased to slightly below normal. Lymph creatinine decreased within 4 hours following irradiation. After 18 hours it rose gradually toward the normal level. Sugar and chloride levels showed no appreciable change. The total number of white blood cells in lymph dropped precipitously within 4 hours after irradiation, and remained at a low level throughout the period of observation. Bacterial cultures of blood and lymph were negative.

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## DISTRIBUTION OF BLOOD IN THE RABBIT'S KIDNEY

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INTEREST in the intrarenal distribution of blood has been stimulated by the work of Trueta and his associates (1), who believe that the kidney has two potential circulations, a greater and a lesser, and that under extreme conditions blood may pass almost exclusively through one or the other. Such uneven distribution of blood was not due to opening of arteriovenous anastomoses described by Spanner (2) and others (3-4) but to diversion of blood to the large juxtamedullary glomeruli by nervous impulses. Unfortunately Trueta did not make any measurements of renal blood flow. That splanchnic stimulation diminishes renal blood flow and that reflex renal vasoconstriction may be induced by stimulation of sensory nerves has been known since the early experiments of Cohnheim and Roy (5), and of Bradford (6).

That variations in renal blood flow might not be distributed uniformly through the kidney was suggested by Hermann (7) and demonstrated in the frog by Richards and Schmidt (8). Variability in the number of glomeruli receiving blood under different conditions was shown in the rabbit by Hayman and Starr (9). White (10), Shannon (11), and Smith (12) do not believe that such variation in the amount of renal tissue perfused occurs in the dog and man, but rather that variation in renal blood flow is uniformly distributed throughout all nephrons.

Before diversion of blood, or by-pass of cortical vessels, can be accepted, it is necessary to show that blood flow through other channels is increased. Diversion of blood from one channel to another is not the same as cessation of flow in one channel while that through another remains unchanged. If diversion of a large fraction of the normal cortical blood flow to other channels occurs, it should be possible to demonstrate cortical ischemia without significant reduction in total renal blood flow.

Trueta's hypotheses have been eagerly adopted by a number of investigators, particularly clinicians, as offering an explanation for the phenomena of essential hypertension, the crush syndrome, cortical necrosis, the 'shock' kidney, and reflex anuria. The conclusions of those who have attempted to confirm Trueta's observations are not uniform. Goodwin, Sloan and Scott (13), and others (14, 15) found India ink distributed as described by Trueta under appropriate conditions. Black and Saunders (16) believed that a rise in  $C_I/C_{PAH}$  with a PAH extraction of less than 80 per cent indicated diversion of blood from cortex to medulla. Using clearance techniques and extraction percentages, Rubi and Schroeder (17) were unable to find evidence of a shunt after injection of adrenaline. Kahn *et al.* (18) were unable to produce the by-pass described by Trueta by adrenaline, renin, or amyl nitrite. Moyer *et al.* (19) could

find no evidence of a renal vascular shunt following stimulation of the sciatic nerve in dogs or rabbits. Renal blood flow decreased, and A-Vo increased, instead of diminishing as suggested by Trueta's observations.

#### PRELIMINARY EXPERIMENTS

Preliminary experiments were made with excised rabbit kidneys to investigate a possible relationship between perfusion pressure and distribution of perfusion mass in absence of neural influence. Rabbits were killed by a blow to the occiput, the kidneys removed immediately and cannulae tied in the renal artery and vein. They were perfused with normal saline at 100 cm. H<sub>2</sub>O pressure until the cortex was pale and the perfusate clear of blood. Then the arterial cannula was perfused with a freshly prepared solution of 2.5 per cent Prussian blue in 6 per cent acacia at a constant pressure which was varied in different experiments from 5 to 100 cm. H<sub>2</sub>O. Perfusion was continued until the colored perfusate appeared in the venous cannula. The kidneys

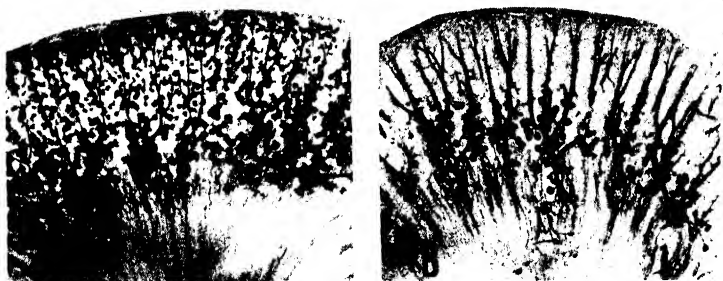


Fig. 1. PHOTOMICROGRAPHS OF excised rabbit kidneys perfused with 2.5 per cent Prussian blue in 6 per cent acacia.  $\times 12$ . A: perfusion pressure 50 cm. H<sub>2</sub>O. Uniform filling of all glomeruli; B: perfusion pressure 25 cm. H<sub>2</sub>O. Filling of juxtamedullary glomeruli and vasa recta only.

were cut across their long axis, fixed in 10 per cent formalin, sectioned at 100 to 150 micra, cleared in glycerine, and mounted in plastic.

Perfusion at pressure of 50 to 100 cm. H<sub>2</sub>O progressed relatively rapidly with venous effluent becoming blue immediately after the cortex became blue. Sections of these kidneys revealed dye in virtually all of the glomeruli (fig. 1A). With perfusion at 20 to 35 cm. H<sub>2</sub>O the cortex remained pale or slowly assumed a mottled blue color and the flow from the venous cannula was very slow. Sections showed the dye in the juxtamedullary glomeruli only (fig. 1B). The cortical glomeruli, except occasionally those deep in the cortex, were never filled although dye was often present in the interlobular arteries extending to the periphery of the cortex. Kidneys perfused at intermediate pressures frequently showed filling of juxtamedullary and deeper cortical glomeruli, while those in the outer part of the cortex were unstained. With perfusion pressure of less than 20-cm. pressure no flow was demonstrable, the dye appearing to diffuse slowly through the vessels giving an irregular distribution of dyed glomeruli. Reduction of the viscosity of the perfusate by substituting aqueous Prussian blue for the Prussian blue-acacia mixture augmented the rate of flow at any given pressure so that at all pressures used there was filling of all glomeruli. If the kidneys were chilled

in an ice box prior to perfusion the resistance to flow appeared to be increased with a corresponding reduction in the rate of flow at any given pressure. Sections of kidneys so treated revealed perfusion of juxtamedullary and deep cortical glomeruli only even at pressure up to 100 cm. H<sub>2</sub>O.

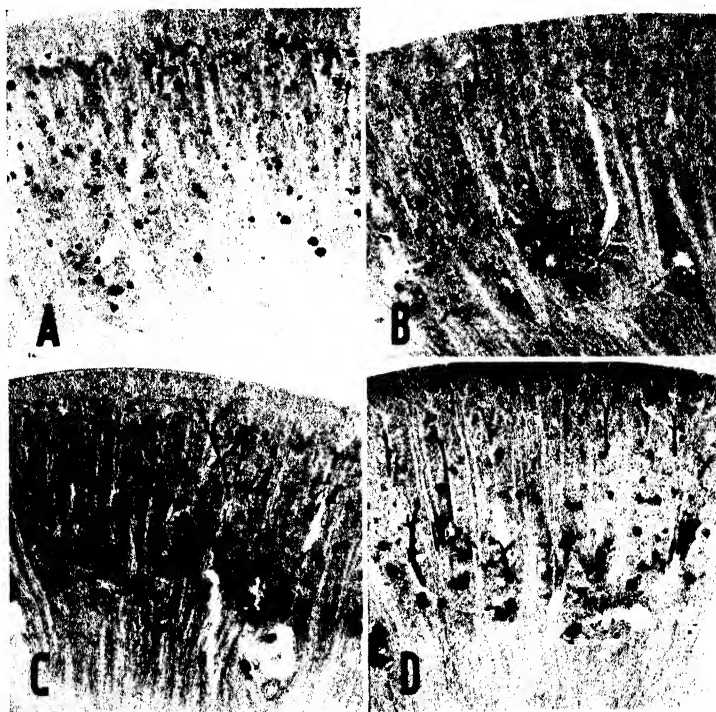


Fig. 2. PHOTOMICROGRAPHS OF distribution of Janus Green after intraarterial injection.  $\times 12$   
A: *exper. 60*. Control. RBV 25 cc/min. B.P. 110 mm. Hg. Uniform staining all glomeruli; B: *exper. 15*. Sciata stimulation. RBF 11 cc/min. B.P. 60 mm. Hg. Staining of deep glomeruli only; C: *exper. 75*. Hemorrhage. RBF 8 cc/min. B.P. 35 mm. Hg. No staining of peripheral glomeruli; D: *exper. 26*. Traction on mesentery. RBF 4 cc/min. B.P. 40 mm. Hg. Spotty staining of cortical glomeruli.

These preliminary experiments indicated that the resistance to blood flow is not equal in all parts of the kidney, but is less in the juxtamedullary glomeruli which arise from the more proximal parts of the intralobular arteries than in the cortical glomeruli whose afferent arterioles are more distal branches. They also showed that it was possible to produce in the excised rabbit kidney the picture described by Trueta of selective perfusion of the juxtamedullary glomeruli when perfusion pressure and rate of flow were low. This emphasized the necessity of obtaining observations on blood pressure and renal blood flow in attempts to confirm Trueta's observations in the living animal.

#### METHODS

Rabbits were anesthetized by intravenous sodium pentobarbital, 20 to 60 mg/kg., occasionally supplemented with ether during the operative procedures. The

gastro-intestinal tract was excised following ligation of its vessels. The abdominal aorta and inferior vena cava were ligated just above their bifurcations. Blood pressure was recorded from a carotid artery by mercury manometer. Renal blood flow through the left kidney was estimated by a modification of Selkurt's (20) method. A rubber T-tube was connected to cannulae in renal vein and inferior cava. An electromagnetic clamp closed the side-arm which when activated opened the side-arm and closed the caval branch, thus diverting renal vein blood into a 10-cc. graduate. Collection periods timed with a stop watch, varied from 5 to 15 seconds. The renal circulation was interrupted for 1 to 2 minutes during insertion of cannulae. Heparin, 2 mg/kg. was used as anticoagulant. Blood was immediately returned through a cannula in an external jugular vein through which an infusion of physiological salt solution, 0.2 to 0.4 cc/kg./minute, was maintained. After a period of control observations the experimental conditions were established. Janus Green (0.5 cc. of a 2.5 per cent solution in physiological saline) was then injected into the stump of the superior mesenteric artery and the aorta immediately clamped. The kidneys were removed as quickly as possible and perfused with salt solution via the renal artery until effluent was clear and then with 5 per cent ammonium molybdate to fix the dye. Sections were examined under a dissecting microscope, then fixed in 10 per cent formalin. Frozen sections cut at 135 were mounted in glycerine or glycerine and plastic. Control experiments revealed no difference in the distribution of dye between the manipulated left kidney and the right. The right kidney served as a control in each experiment. That the vasoconstrictor action of the dye does not interfere with staining of glomeruli receiving blood at the time of its injection was shown by Hayman and Starr. The presence of any dye in a glomerular capillary was considered evidence that that glomerulus was being perfused at the time of dye injection. In the same kidney the number of capillaries stained in a glomerulus might vary from a few to all. In general, all of the capillaries of the deep cortical or juxtamedullary glomeruli appeared well stained while varying proportions of the capillaries of the more peripheral glomeruli were stained, depending upon the experimental conditions, but regardless of their position in the cortex.

#### RESULTS

The results of these experiments are summarized in table 1. The first figures under renal blood flow and blood pressure are the values during the control period, and the second figures those at the time the dye was injected. Kidney weights are not included since the variable degrees of edema acquitted during perfusion rendered them meaningless.

In the first group of 3 experiments the renal blood flow varied from 25 to 40 cc. per minute and the carotid blood pressure from 55 to 110 mm. Hg. at the time of dye injection. The average values are 31 cc. and 86 mm. Hg. The dye was uniformly distributed throughout the cortex, almost all the glomeruli both juxtamedullary and cortical being well stained. The rate of saline infusion and urine formation were somewhat greater in these experiments than in others.

In the second group of 7 experiments the distribution of the dye corresponded to that described by Trueta as indicative of a cortical by-pass, or diversion of blood flow through the juxtamedullary glomeruli. This followed stimulation of the central end of the cut sciatic nerve, stimulation of the nerve fibers around the renal artery, or



hemorrhage. The renal blood flow at the time of dye injection varied from 8 to 18 cc. per minute, averaging 10.7 cc. Carotid blood pressure varied from 30 to 95 mm. Hg, averaging 56 mm. Hg. Sections showed that the deep glomeruli were uniformly stained, while in the more peripheral parts of the cortex either no dye at all could be detected, or a few scattered glomeruli showed faint staining, frequently only a 'button' at the point of entrance of the afferent artery (fig. 2).

The next 5 experiments in the table represent four unsuccessful attempts to alter the distribution of blood in the kidney by stimulation of nerves and one in which

TABLE 1. RELATION OF DISTRIBUTION OF JANUS GREEN B AFTER INTRA-ARTERIAL INJECTION TO RENAL BLOOD FLOW AND SYSTEMIC BLOOD PRESSURE IN RABBITS

EXPER. NO.	WT.	LEFT RENAL BLOOD FLOW	BLOOD PRESSURE	GLOMERULAR STAINING		REMARKS
				Deep	Cortex	
	kg.	cc/min.	mm. Hg			
18	3.0	25-29	50-55	Uniform	Uniform	Control
21	2.5	37-40	85-95	Uniform	Uniform	Control
69	2.7	35-25	100-110	Uniform	Uniform	Control
15	3.1	32-11	60-60	Uniform	None	Sciatic stimulation
77	3.2	17-10	70-40	Uniform	Poor	Sciatic stimulation
27	3.0	28-18	65-40	Uneven	Poor	Stim. perirenal plexus
70	2.6	24-9	95-90	Uneven	None	Stim. perirenal plexus
39	3.9	28-10	115-30	Uniform	None	Hemorrhage
75	2.8	15-8	80-35	Uniform	None	Hemorrhage
34	2.6	16-9	110-95	Uniform	None	Spontaneous
22	3.2	44-18	95-80	Uniform	Uniform	Stim. perirenal plexus
14	2.8	30-3	100-100	Uniform	Uniform	Stim. perirenal plexus
29	3.8	24-17	120-85	Uniform	Uniform	Sciatic stimulation
76	2.5	40-29	100-70	Uniform	Uniform	Sciatic stimulation
28	2.4	32-10	90-85	Uniform	Uniform	Spontaneous
19	2.8	26-22	90-80	Uniform	Segmental	Mesenteric traction
26	2.8	20-4	90-40	Uniform	Spotty	Mesenteric traction
73	2.3	12-3	80-35	Spotty	Spotty	After caffeine

renal blood flow fell for some unapparent reason. In 3 of the 5 experiments renal blood flow is only moderately reduced. In the other 2 experiments renal blood flow was markedly reduced, but the blood pressure was maintained at a high level. These 2 experiments may be regarded as indicating that even when total renal blood flow is markedly reduced, blood may be distributed to all parts of the kidney provided systemic blood pressure is adequate.

In the 3 other experiments summarized in the table, the dye was irregularly distributed. In 2 blood flow fell during traction on the root of the mesentery, and in the 3rd following a large dose of caffeine in an attempt to produce a diversion of blood from juxtamedullary to cortical glomeruli. The first 2 showed uniform filling of the deep glomeruli, while staining of those in the more peripheral part of the cortex was

irregular. In *experiment 19* it appeared as if those arising from one intralobular artery were filled, while those arising from another intralobular artery, except for the most proximal, were unstained. In *experiment 73* staining of both deep and superficial glomeruli was spotty and irregular. It was not possible to determine whether those that were stained arose from the same or different intralobular arteries.

#### DISCUSSION

Staining of peripheral glomeruli without staining of the deeper or juxtamedullary glomeruli did not occur in any of our experiments. Thus no evidence was obtained that blood flow might be diverted from a 'medullary' to a 'cortical' pathway. Following sciatic nerve stimulation, stimulation of renal artery, or hemorrhage, the distribution of dye in some experiments indicated that only or chiefly the deeper or juxtamedullary glomeruli were perfused with blood at the time the dye was injected. This distribution only occurred when renal blood flow was reduced but was apparently independent of systemic blood pressure over a range of 30 to 95 mm. Hg. On the other hand (*experiments 14* and *28*) it did not always occur when renal flow was low provided systemic blood pressure was well maintained. Since in those experiments in which the cortex appeared to be ischemic, renal blood flow had been reduced to from 33 to 64 per cent (average 48%) of the control flows it does not seem appropriate to regard this as a diversion of blood from cortex to medulla, or of a cortical by-pass. That the blood flow per perfused glomerulus may not have been increased cannot be determined from these experiments.

There is probably more than one mechanism accounting for the favorable status of the juxtamedullary glomeruli. Perfusion of excised kidneys showed a definite relationship between perfusion pressure and the distribution of glomerular filling, in that at low pressures and consequent small volume flow, the juxtamedullary glomeruli were selectively perfused. Apparently the resistance offered by the larger juxtamedullary glomeruli with large efferent vessels and the arrangement of their postglomerular vessels is less than that offered by the glomeruli in the more peripheral parts of the cortex. This may be an important factor in the distribution of stained glomeruli after hemorrhage when both renal blood flow and systemic blood pressure were low.

Constriction of intralobular arterioles following sympathetic nerve stimulation may not be uniform throughout their length. Possibly the distal portion is more liberally supplied with nerve fibers. Even if the degree of shortening of muscle fibers is the same throughout the arteriole, the wrinkling of the endothelium accompanying contraction would produce greater obstruction to blood flow in the smaller distal portion than in the larger more proximal portion. The filling of intralobular arterioles without staining of the more peripheral afferent arterioles and glomeruli suggests that the afferent arteriole is the point where blood flow is usually shut off. Closure of peripheral afferent arterioles while the more proximal are still perfused may be due to either of the possibilities mentioned, or to a combination of both. Pressure, too, is higher in the proximal than distal part of the intralobular arteriole. The view that a given vasoconstrictor stimulus produces a greater increase in resistance to blood flow in small arterioles than in those slightly larger is consistent with Richards, Barnwell

and Bradley's (21) demonstration that a minute amount of adrenaline caused swelling of the glomerular tuft. The irregular and segmental distribution of stained glomeruli in some preparations suggests that under certain conditions constriction may be more marked in some branches of the renal artery than in others, rather than in the distal parts of all.

These experiments are not believed to lend any support to the hypothesis that there are two circulations through the kidney, and that renal blood flow may be diverted from one to the other. Rather, they indicate that when renal blood flow is reduced, the reduction may not be uniform throughout the kidney, but that it may be greater in the peripheral cortical glomeruli than in those situated more deeply toward the medulla. This is of importance in understanding the phenomena of the crush syndrome, the 'shock' kidney and bilateral cortical necrosis. Selkurt (22) showed that after a period of renal ischemia kidney function as measured by clearances did not return promptly with re-establishment of renal blood flow, and Van Slyke (23) found that if renal ischemia was prolonged recovery of kidney function did not occur and the animals died in uremia.

#### SUMMARY

Attempts to confirm Trueta's hypothesis that renal blood flow may be diverted from cortical to medullary channels under certain conditions are described. When excised rabbit kidneys were perfused with Prussian blue in 6 per cent acacia solution at low pressures, only the deep juxtamedullary glomeruli were filled, while with higher pressures all glomeruli were filled.

Renal blood flow and systemic blood pressure were measured in anaesthetized eviscerated rabbits, and the distribution of glomeruli receiving blood under various conditions shown by staining with Janus Green B after intra-arterial injection. The deeper glomeruli were never free from dye when the peripheral ones were stained. In some experiments, following sciatic stimulation, stimulation of the perirenal plexus, or hemorrhage the juxtamedullary glomeruli were uniformly stained while those in the peripheral areas of the cortex were unstained. This distribution, which resembled that described by Trueta, only occurred when renal blood flow was low.

These experiments furnish no support for the hypothesis that any significant quantity of blood is diverted from cortical to medullary pathways by direct or reflex stimulation of renal vasoconstrictor nerves.

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# MECHANISM BY WHICH ACTH INCREASES THE EXCRETION OF URATE<sup>1</sup>

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**F**ORSHAM *et al.* (1) first observed that an increased excretion of urate occurred in man after the administration of pituitary adrenocorticotrophic hormone (ACTH). It has not been determined, however, whether this increased urate excretion results from an increased production of urate in the body after ACTH or whether it is due to some change in the renal excretion of urate. As Thorn *et al.* (2) demonstrated, no essential change occurred in the glomerular filtration rate as measured by the creatinine excretion after injection of ACTH so that if increased urate excretion is due to a renal effect of ACTH, it must be the tubular component of the kidney involved. Recently Ingbar *et al.* (3) found that both the inulin and hippurate clearances increased in man after the administration of large amounts of ACTH, but were unchanged if moderate amounts were given. These same investigators likewise found that the increase in urate excretion after administration of ACTH was paralleled by a decrease in plasma urate. This last finding, of course, suggests that ACTH does not increase the production, but the renal excretion of urate. The observations of Benedict *et al.* (4) also suggest that ACTH increases the renal excretion of urate.

In order to determine the manner in which ACTH effects increased urate excretion, we have analyzed the allantoin and urate excretion of the rat after administration of ACTH. If ACTH increases purine metabolism, allantoin and not urate should be increased in the rat's urine, since urate, even when produced in tremendous excess, is changed rapidly into allantoin (5). If ACTH solely effects an increased renal excretion of urate, only this substance, and not allantoin, should be increased in the urine of the treated rat. Nephrectomized rats were given ACTH and the urate and allantoin accumulation in their plasma was compared to that of untreated nephrectomized rats in order to determine if ACTH effected an increase in rate of purine accumulation. The results of these studies indicated that ACTH had no discernible effect upon 'total purine' (uric acid plus allantoin calculated as uric acid) production, but acted upon the renal excretion of urate.

## METHODS

*Renal Excretion Studies.* Nine male albino rats were individually caged and fed only 5 per cent glucose solution. Twenty-four hours later, a blood sample was taken

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and each rat received one mg. of ACTH (Armour) intramuscularly. Urine was collected daily from these rats for 48 hours and a second blood sample was obtained 24 hours after the injection of ACTH. Blood plasma samples and urine samples were analyzed for allantoin, urate and total purine according to methods previously described (5). Urine samples were analyzed for creatinine according to the method of Folin and Wu (6). In our hands, replicate analyses for uric acid differ from their means by an average of 1.4 per cent of the mean value. Eight control rats similarly were studied.

*Nephrectomy Studies.* Fourteen albino rats were nephrectomized and given 5 mg. of ACTH (Armour). Blood samples were obtained before and 24 hours after operation in all of the rats. Blood samples also were obtained from 8 of these rats, 4 hours after nephrectomy. The plasma allantoin, urate and total purine of each

TABLE 1. EFFECT OF ACTH UPON URATE AND ALLANTOIN CLEARANCE OF THE RAT<sup>1</sup>

TABLE I. URINE OF RATS											
NO. OF RATS	WT.	URINE I (0-24 HRS.)					URINE II (24-48 HRS.)				
		U.V.	Urate	All.	T.P.	Creat.	U.V.	Urate	All.	T.P.	Creat.
		cc.	mg.	mg.	mg.	mg.	cc.	mg.	mg.	mg.	mg.
<i>Rats given 1 mg. of ACTH</i>											
9	Av. 177	70	2.98	33.1	38.4	5.89	121	2.82	33.4	38.2	5.74
	S.E. mean:		0.211					0.157			
<i>Control Rats</i>											
8	Av. 177	77	2.45	34.1	38.4	5.74	91	2.51	33.3	37.9	5.86
	S.E. mean:		0.123					0.112			

<sup>1</sup> Tables 1 and 2 with detailed data have been deposited with the American Documentation Institute, 1719 N Street, N.W., Washington 6, D. C. For copies of these tables order Document 2924 directly from the American Documentation Institute, remitting \$0.50 for microfilm (images 1 inch high on 35-mm. film) or \$0.50 for photocopies (6 x 8 inches).

U.V.—Urine volume. All.—Allantoin. T.P.—Total purine. Creat.—Creatinine.

sample were determined. Twelve control nephrectomized rats were studied in the same manner.

## RESULTS

*Renal Excretion of Urate and Allantoin After ACTH.* ACTH in the dosage used effected no essential change (see table 1) in the creatinine excretion of the rats, suggesting that no change in the rate of glomerular filtration had occurred in the 48 hours following its injection. Likewise, no significant change occurred in the allantoin excretion. The average excretion of allantoin for the 24-hour period after injection was 33.1 mg. per day (range: 24.4-42.8 mg.) as compared to 34.1 mg. (range 22.0-45.8 mg.) in the controls. Similarly, no increase in allantoin excretion was noted during the second 24-hour period after injection.

However, the treated rats did show an increase in urate excretion. This average excretion was 2.98 mg. (range: 2.09-40.7 mg.) for the first 24 hours as compared to 2.45 mg. (range: 1.73-2.82 mg.) in the controls—an increase above the control ex-

cretion of approximately 18 per cent. The odds against this difference occurring by chance are greater than 60 to 1. The urate excretion during the second 24-hour period was 2.82 mg. (range: 2.27-3.76 mg.) in the treated rats and 2.51 mg. (range: 1.65-2.92 mg.) in the controls—an increase of approximately 11 per cent. However, this increase was not considered significant, since the odds against the observed difference occurring by chance are only 5 to 1. Similarly, no significant increase was observed (see table 1) in the excretion of 'total purine' after injection of ACTH.

*Accumulation of Urate and Allantoin in Plasma of Nephrectomized Rats After ACTH.* As table 2 indicates, ACTH provoked no greater rise in the plasma urate of nephrectomized rats than that observed in the control, untreated nephrectomized rats. Although in both series the average urate concentration of plasma rose 25 per cent after 4 hours only to return to pre-operative levels 24 hours after nephrectomy, 4 of the 8 rats receiving ACTH and 2 of the 6 controls had a lower urate concentra-

TABLE 2. EFFECT OF ACTH UPON PLASMA URATE AND ALLANTOIN ACCUMULATION IN NEPHRECTOMIZED RATS<sup>1</sup>

NO. OF RATS	WT.	BEFORE NEPHRECTOMY			AFTER NEPHRECTOMY (4 HRS.)			AFTER NEPHRECTOMY (24 HRS.)		
		UR	All.	T.P.	UR	All.	T.P.	UR	All.	T.P.
Rats given 5 mg. of ACTH										
14	Av.: 159	1.18	3.69	5.08	1.51	19.7	22.3	1.17	46.5	50.5
	S.E. mean:	0.104		0.231	0.158			0.112		
Control Rats										
12	Av.: 161	1.38	3.25	4.57	1.79	20.0	23.0	1.15	46.8	51.4
	S.E. mean:	0.156		0.361	0.095			0.066		

<sup>1</sup> See footnote 1, table 1.

UR—Urate in mg/100 cc. All.—Allantoin in mg/100 cc. T.P.—Total purine in mg/100 cc. = uric acid plus allantoin (expressed as the equivalent amount of uric acid).

tion at 4 hours. The rise in the average allantoin content of plasma was almost identical (see table 2) in the treated and control nephrectomized rats, both 4 and 24 hours after nephrectomy. Total purine accumulation 4 and 24 hours after nephrectomy was essentially the same in both series of rats.

#### DISCUSSION

The moderate increase in urate excretion observed in our rats for 24 hours after administration of ACTH indicates that the same mechanism causing an increase in urate excretion in man is also operative in the rat.

The failure of the allantoin excretion to increase after administration of ACTH strongly suggests that no change has been effected in the rate of purine production in the rat since allantoin is the chief end product of purine metabolism in this species. Moreover, the failure of ACTH to effect a change in the usual rate of accumulation of urate and allantoin in the plasma of rats after nephrectomy points again to the refractoriness of normal purine metabolism to the acute effects of this hormone.

The increased excretion of urate in rats receiving ACTH, then, in the absence of any change in purine metabolism suggests that the phenomenon is solely one resulting from the effect of ACTH administration on the kidney. Furthermore, the failure of both allantoin and creatinine excretion to change after ACTH indicates that the increase in urate excretion could not be due to changes in rate of glomerular filtration, since both the excretion of allantoin (7) and creatinine are dependent upon glomerular filtration. It would therefore appear that ACTH in some manner impedes the tubular reabsorption of urate. It is of interest in this connection that Ingbar *et al.* (3) have shown that administration of large amounts of ACTH to man may effect changes in tubular function.

#### SUMMARY

The administration of ACTH to rats caused a moderate increase in urate excretion with no observable change in allantoin or creatinine excretion. Administration of ACTH to nephrectomized rats did not effect a change in the rate of urate or allantoin accumulation. The results obtained indicate that the administration of ACTH has no effect on the purine metabolism of the rat, but probably has a direct effect upon the tubular reabsorption of urate.

The authors wish to express their thanks to Mary Alice Garms for her technical assistance.

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# FACTORS INFLUENCING BILE FLOW IN THE DOG AND RAT

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THE evaluation of hepatic secretory function by use of the biliary fistula dog is complicated by the variability of bile flow in this preparation. To pre-existing factors of spontaneous variability are added an anesthetic agent and a surgical procedure, both of which may cause irregular degrees of hepatobiliary depression or stimulation (1, 2). The reportedly lower variation in the bile-flow response to sodium dehydrocholate as compared with the control rate of flow (3) may reflect the nullification of such sources of variation by substitution of a relatively intense, fixed stimulus.

The objective of the present study was to determine the influence of various somatic factors on bile secretion under basal conditions and in response to dehydrocholic acid. These factors include sex, surface area, body weight, liver weight and bile depletion. The effects of species differences on certain of those factors were estimated by comparisons between the dog and the rat.

## METHODS AND MATERIALS

Dogs fasted for 18 hours were anesthetized with sodium pentobarbital. The cystic duct was clamped by a hemostat and the common bile duct cannulated. The cannula was connected to a 3-mm. plastic tube brought through an abdominal incision. The open end of the plastic tube was maintained at or slightly below the level of the common bile duct. Bile flow was measured at 30-minute intervals; collections were pooled hourly and aliquots analyzed for total solid content. No bile was returned to the animals. Dehydrocholic acid, when given, was administered by intravenous injection following two one-half-hour periods. The acid was made up in a 2 per cent solution by adding sufficient sodium hydroxide to convert it to the sodium salt.

Rats, 225 to 410 gm. in weight, obtained from Sprague-Dawley, were anesthetized with sodium pentobarbital. Bile flow was measured using the same technic described for the dog, except that collection was made into 2-cc. tared ampules. The output was measured at one-hour intervals by weighing.

The existence of a linear relationship between factors was determined by calculation of the correlation coefficient ( $r_{29}$ ). The significance of correlation coefficients and of differences between means was estimated by calculation of the  $t$  and  $P$  values.

## RESULTS

*Dogs.* The basal rate of bile flow (cc/dog/hr.) in a series of 260 dogs did not differ significantly for male and female animals. The rate did not correlate significantly

with surface area or body weight (table 1 and fig. 1) although other investigators have reported a low but significant correlation with body weight under certain circum-

TABLE 1. COMPARISON OF VARIOUS PARAMETERS IN DOGS AND RATS

RELATION	DOG			RAT		
	df	$r_{xy}$	$P$	df	$r_{xy}$	$P$
Basal bile flow: body weight	258	0.080	$>0.05$	137	0.599	$<0.01$
Basal bile flow: surface area	258	0.079	$>0.05$			
Basal bile flow: liver weight	36	0.212	$>0.05$	194	0.590	$<0.01$
Body weight: liver weight	36	0.723	$<0.01$	194	0.750	$<0.01$

stances (4). An obscuring influence of seasonal variation on the latter relation was considered; however, basal rate of bile flow still did not correlate significantly with body weight in dogs with similar average flow rates studied during 5 consecutive months.

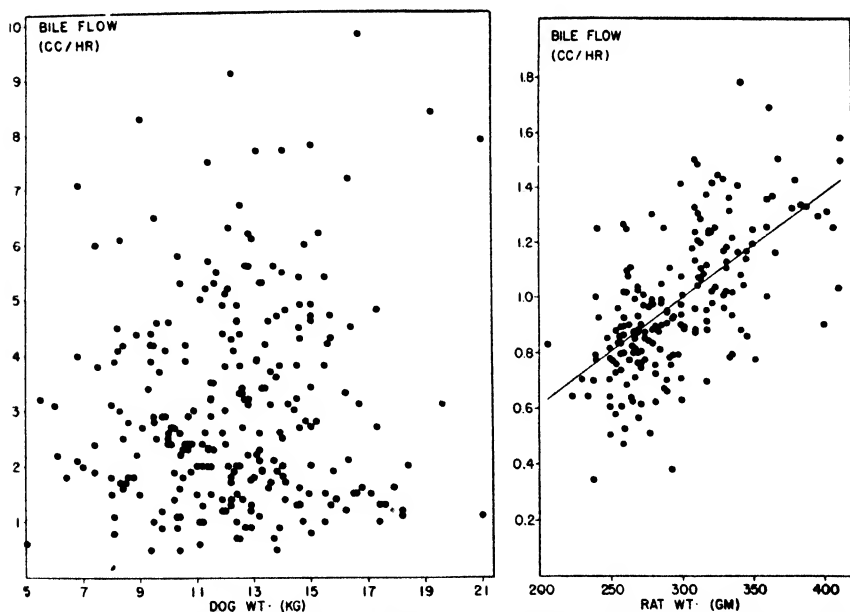


Fig. 1. RELATION OF BASAL BILE-FLOW RATE (cc/hr.) to body weight in 260 dogs ( $r_{xy} = 0.080$ ) and 196 rats ( $r_{xy} = 0.599$ ). Intercept of regression line:  $a = -0.17$ ; slope:  $b = 0.0039$  (by method of least squares).

The average bile-flow rate was estimated in relation to both body and liver weights in a series of 38 dogs. The hourly rate was  $0.26 \pm 0.024$  cc/kg. of body weight

TABLE 2. BILE SECRETION IN NINE DOGS (MEAN VALUES)

HOURS	1	2	3	4	5	6	7	8	9	10
Volume, cc/kg.	0.26	0.23	0.26	0.24	0.22	0.25	0.24	0.26	0.23	0.23
Total solids, mg/kg.	25.8	19.3	17.1	13.6	12.1	14.1	11.9	11.8	9.8	7.7
gm/100 cc.	15.4	10.6	8.8	8.2	8.2	7.6	8.9	8.3	6.0	6.0

and  $0.64 \pm 0.201$  cc/100 gm. of liver. In this smaller series basal bile flow correlated neither with body weight nor liver weight; the latter two parameters themselves were well correlated (table 1).

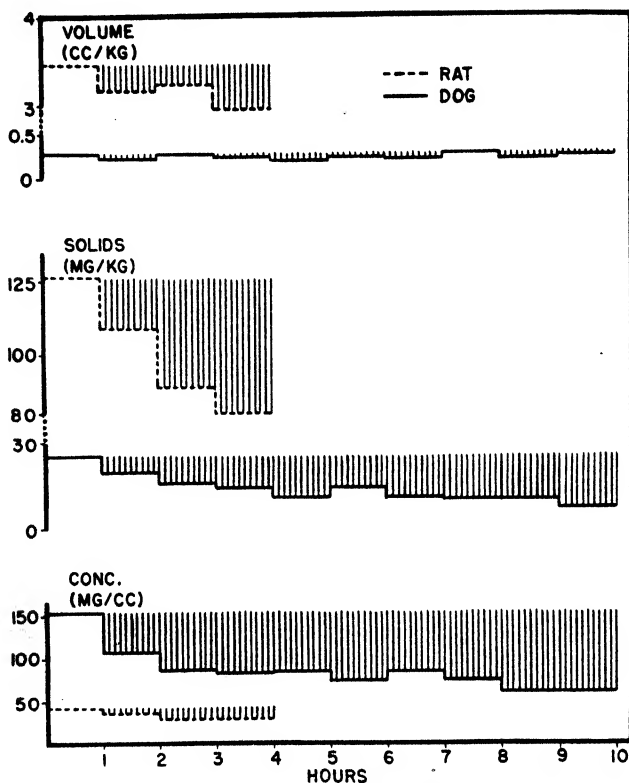


Fig. 2. RELATION OF BILE-FLOW RATE, total solid excretion and total solid concentration to time in 9 dogs and 11 rats. No bile returned to animals.

The rate of bile flow was measured for 10 hours in 9 dogs (table 2). The average rate of flow did not vary significantly with time. The total solid excretion and the concentration of solids showed a definite regression (fig. 2). The fall in solid excretion

was interpreted to be the result of continuous removal and non-replacement of bile constituents during the experiment. This trend may become important when determining whether a choleric agent increases secretion of both water and solids (cholesteris) or water only (hydrocholeris).

Dehydrocholic acid, 20 mg/kg., was administered to 86 dogs. The absolute bile output for the 30-minute period following administration correlated linearly ( $r_{xy} = 0.532$ ;  $P < 0.01$ ) with the control flow for the preceding half hour (fig. 3). The response also correlated with total dose ( $r_{xy} = 0.560$ ;  $P < 0.01$ ) (fig. 4). Since total dose was determined by body weight, the response was necessarily correlated with weight also under the specific conditions of dosage in these animals. The absence of a general

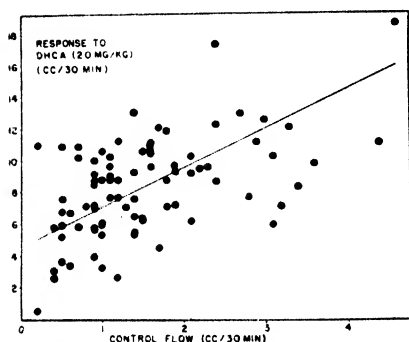


Fig. 3 (left). RELATION OF CONTROL FLOW to bile-flow response after dehydrocholic acid (20 mg/kg.) in 86 dogs. Intercept of regression line:  $a = 4.59$ ; slope:  $b = 2.469$  (by method of least squares).

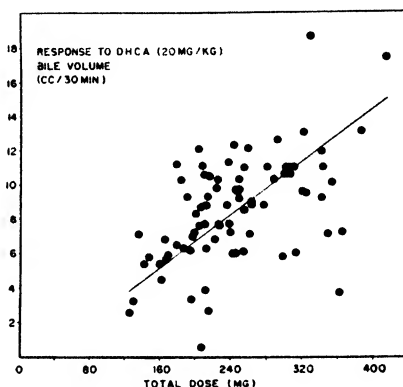


Fig. 4 (right). RELATION OF TOTAL DOSE to bile-flow response after dehydrocholic acid (20 mg/kg.) in 86 dogs. Intercept of regression line:  $a = -0.91$ ; slope:  $b = 0.038$  (by method of least squares).

relation of response to body weight per se was indicated by the results of the following experiments:

Bile output of the 8 largest dogs in the above series was compared with the output of 8 small dogs given approximately the same total dose (table 3, group A). Similarly, the 8 smallest dogs in the original series were compared with 8 large dogs (table 3, group B). The mean control bile flows for the large and small dogs did not vary significantly. Comparison of the bile-volume response with the total dose demonstrated that large and small dogs secrete amounts of bile which were not significantly different when given similar total doses of dehydrocholic acid. This finding was interpreted to mean that the choleric response of the liver was dependent upon the total quantity of dehydrocholic acid administered.

As a consequence of the dependency of choleric response upon total dose it appeared likely that the slope of dose-response curves based upon mg/kg. relationships (3) would vary with the weight of the animals used. In support of this hypothesis dose-response relationships were determined in the two additional groups of 8 dogs

each used in the above experiment. The weight of the first group averaged 7.9 kg. and the second averaged 18.3 kg. The control bile-flow rates of the two groups did not differ significantly. The groups were given dehydrocholic acid in doses of 7, 14, 28 and

TABLE 3. BILE-FLOW RESPONSE IN LARGE AND SMALL DOGS TO APPROXIMATELY EQUAL DOSES OF DEHYDROCHOLIC ACID

GROUP	NO. OF DOGS	MEAN WT., KG. (RANGE)	AV. TOTAL DOSE, MG.	MEAN BILE OUTPUT		
				cc/30 min. $\pm$ S.E.	<i>t</i>	<i>P</i>
A	8	18.4 (17.3-21.0)	368	10.19 $\pm$ 3.85	0.5215	0.5
	8	7.9 (7.2-8.6)	444	10.59 $\pm$ 0.767		
B	8	18.3 (16.3-21.4)	128	6.54 $\pm$ 0.76	1.4178	0.2
	8	7.3 (6.3-8.3)	146	5.21 $\pm$ 0.55		

56 mg/kg. in random order. Bile output during the first 30 minutes after injection was used as a measure of the response. Bile flow was allowed to return to the control rate before the next injection. Calculation of the average dose-response curves for the two groups demonstrated the slope for the group of larger animals to be approximately

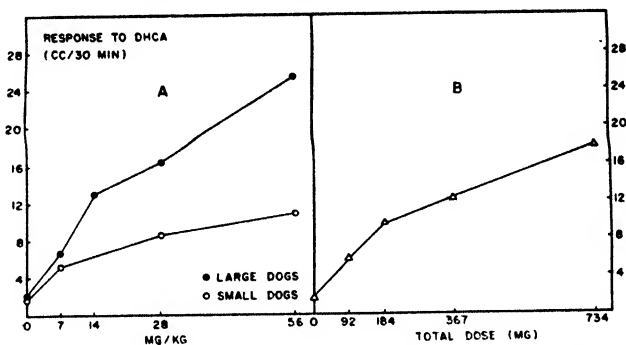


Fig. 5. A: RELATION OF BILE-FLOW response to dose per unit body weight of dehydrocholic acid in 8 large and 8 small dogs; B: relation of bile-flow response to total dose of dehydrocholic acid in the 16 dogs from A.

twice as steep as that for the group of smaller animals despite identical doses per unit of body weight (fig. 5A).

It was concluded from these data that dose-response curves for choleric agents of the dehydrocholic acid type must be based upon total doses rather than on doses per unit of body weight in order to be of general validity in the evaluation of choleric

activity. The total dose-response curve based on the combined data from the 16 dogs in the two groups described above is illustrated in figure 5B.

*Rats.* The basal rate of bile flow (cc/hr.) was found to correlate significantly with body weight (table 1 and fig. 1) and with liver weight (table 1) in a series of 196 rats. Body weight and liver weight were well correlated (table 1). No significant difference between males and females was demonstrated. The average bile-flow rate, as determined in 11 rats, was  $3.47 \pm 0.187$  cc/hour/kg. body weight and  $9.9 \pm 0.45$  cc/hour/100 gm. of liver.

The rate of bile flow was measured for 4 hours in 11 rats. The average rate of flow, total solid excretion and concentration of solids all decreased with time (fig. 2).

The total dose-response relationship for dehydrocholic acid was determined by using a group of 4 rats at intravenous dose levels which averaged 50, 100 and 215 mg. These doses in terms of body weight were 191, 382 and 765 mg/kg., corresponding to one-eighth, one-quarter and one-half of the intraperitoneal mouse LD<sub>50</sub>. Bile was col-

TABLE 4. PERCENTAGE RESPONSE OF BILE SECRETION TO DEHYDROCHOLIC ACID IN DOGS AND RATS

DOG		RAT	
Dehydrocholic acid mean total dose	Mean response	Dehydrocholic acid mean total dose	Mean response
mg.	per cent	mg.	per cent
0	100	0	100
92	239	50	256
184	339	100	292
367	448	215	301
734	658		

lected for one hour. The flow rate (cc/kg/hr.) was converted to mean response at each dose level (table 4).

#### DISCUSSION

The rate of bile secretion in the rat was found to be approximately fifteen times that of the dog when body weight or liver weight of the two species was considered. Probably as a consequence of this relatively more rapid depletion of bile, the regression of bile volume and solids with time was much steeper for the rat than for the dog (fig. 2). The absence of a gall bladder in the rat may account for the finding that rat bile was about one-fifth less concentrated than dog bile. The lack of correlation between basal bile flow and either body or liver weight in the dog as compared to the rat could not be explained. It is noteworthy that the percentage response to similar total doses of dehydrocholic acid was of the same order of magnitude in the two species (table 4).

#### SUMMARY

The influence of various somatic factors on bile flow, bile solid excretion, and response to dehydrocholic acid were studied in 276 acute biliary fistula dogs. These

factors included sex, surface area, body weight and bile depletion. The effects of species differences on certain of these factors were estimated by comparisons between the dog and the rat.

In the dog, the basal rate of bile flow did not correlate with either surface area or body weight. The average rate of bile flow remained relatively constant for a period of 10 hours. The rate of total solid excretion and concentration of solids decreased with time. The response to dehydrocholic acid (20 mg/kg. by vein) correlated linearly with the control level of flow and with total dose. Comparison of the effects of dehydrocholic acid in dogs of varying weight indicated that the choleric response of the liver depends on the total quantity administered rather than on the dose per unit weight.

In the rat, unlike the dog, basal rate of bile flow showed significant correlation both with liver and total body weight. The average rate of bile flow, total biliary solids excretion and the concentration of solids all decreased with time.

The percentage response to similar total doses of dehydrocholic acid was similar in the dog and the rat.

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# THE AMERICAN PHYSIOLOGICAL SOCIETY PROCEEDINGS

## FALL MEETING

*Columbus, Ohio, September 13-16, 1950*

### ABSTRACTS OF PAPERS READ

*An asterisk following an author's name denotes "by invitation"*

#### *Cardiac responses to hypothermia in infant mammals.*

E. F. ADOLPH. Univ. of Rochester, Rochester, N. Y.

The object was to characterize responses which change during development of the individual. The relation of heart-beat frequencies to colonic temperatures was thus analyzed. Each individual of known age was immobilized on a frame or in a tube, surrounded by water of controlled temperature. Thermocouples were retained in colon, and often in gullet. Subcutaneous electrodes led to an electrocardiograph. Golden hamsters soon after birth diminished their heart frequencies with cooling, along a concave curve from 35° to 2° C.; cooling below 1° C. still allowed recovery. By 11 days of age this curve became almost linear. At 16 days the lowest part of the curve was lost and the heart stopped irreversibly at 3° C. In the adult, recovery of heart was impossible after it ceased to beat; and at 37° C. the beat was the same as or slower than at 34° C. Cats and rats were studied in the same manner. The 3 species showed similar, but quantitatively different, patterns of cardiac frequencies at low temperatures. No one type of equation could represent the relations in any species at all ages. Some of the differences, such as the restriction of the lower temperature limit, and the  $\Delta$  frequency per  $\Delta$  temperature, are inherent in the heart, since isolated hearts of diverse ages show them. Others may depend upon the maturation of specific transmitted responses, particularly those concerned with increasing the heat production in response to cooling. Such modifications with age represent the progressive realization of specific physiological regulations.

*Is there a respiratory pace-maker?* ROBERT S. ALEXANDER AND SAMUEL L. THORPE. Med. Research Lab., Brecksville Veterans Administration Hospital, Brecksville, Ohio.

Incidental to routine breathing tests, spirometric tracings were obtained from patients instructed to "Breathe in and out as deeply as you can" and then to

"Breathe in and out as rapidly as you can." After 30 to 60 seconds of each period of abnormal breathing, the patients were commanded, "Now breathe naturally." Records of 100 patients who yielded uniform tracings were analyzed with particular attention to the recovery following the periods of voluntary hyperventilation. Of the 200 tests comprising 100 deep and 100 rapid breathing records, 165 exhibited a recovery characterized by some persisting hyperventilation. After the deep breathing period, respiration remained deeper and somewhat slower. After rapid breathing, a persisting acceleration in rate was observed in 90 per cent of the cases. Changes in depth following rapid breathing, however, were strictly random and showed no tendency to correlate with the tidal volume during rapid breathing. It is assumed that these recovery responses represent a residual excitatory state in components of the respiratory center. The data are therefore interpreted as indicating that the respiratory center includes components capable of governing rate independent of depth. This relationship is not compatible with those theories of respiratory center function which postulate respiratory rhythm to be the resultant of the reciprocal action of extrinsic stimuli. It is therefore suggested that more consideration should be given to the possibility that the respiratory center incorporates an intrinsic pace-maker mechanism. Such an hypothesis could aid in explaining the paroxysmal dyspnea observed clinically.

*Some effects of b-diethylaminoethyl xanthine-g-carboxylate methobromide (banthine) on canine pancreatic secretion.* DAVID ANNIS AND GEORGE A. HALLENBECK. Mayo Foundation and Div. of Surgery, Mayo Clinic, Rochester, Minn.

Banthine is primarily an anticholinergic drug. Experiments were designed to observe the effect of banthine, given orally and intravenously, on the external secretory response of the pancreas to a meal of 200 gm. of



meat. The effects of the drug on the secretin mechanism also were studied. Pancreatic juice was collected from 8 dogs by the method of Thomas. Control data were obtained on the normal pancreatic response to the meal; juice was collected every 15 minutes for 3 hours after feeding and measurements were made of volume and the content of alkali and trypsin. These experiments were repeated when banthine had been given i.v. in doses of 0.5 mg. and 5.0 mg/kg. of body weight and by stomach tube in doses of 5.0 mg. and 10.0 mg/kg. To observe the effects of banthine on the secretin mechanism, control data were established based on the volume and alkali and trypsin content of pancreatic juice produced by 15 units of secretin administered i.v. and 30 cc. of tenth-normal hydrochloric acid given intraduodenally. The experiments were repeated after banthine was given in doses of 0.33 to 0.66 mg. and 5.0 mg/kg. of body weight. Banthine administered orally and i.v. reduced the 3 hour total volume and alkali content of pancreatic juice secreted in response to a meal. The trypsin content was reduced only with a dosage which produced tachycardia and other side effects. Banthine had little effect on the volume of juice resulting from injections of secretin or intraduodenal administrations of hydrochloric acid, but the enzyme content was reduced.

*On the mechanism of the electrical silence following cerebellar stimulation.* A. ARDUINI, G. MORUZZI AND C. TERZUOLO (introduced by JOHN M. BROOKHART). Institute di Fisiologia, Università di Pisa, Italy.

Using cats decerebrated at the intercollicular level, the anterior lobe of the cerebellum or the bulbar inhibitory reticular formation were stimulated electrically for 30 sec. using one msec. rectangular pulses at a frequency of 280/sec. Stimuli yielding complete inhibition of decerebrate rigidity and myotatic reflexes were never followed by cessation of electrical activity in the stimulated area of the cerebellum or neighboring regions of the anterior lobe. A short lasting localized electrical silence was obtained only after cerebellar stimuli which were definitely supramaximal for the inhibition of myotatic reflexes and tonus. This effect of supramaximal stimulation was not prevented by mid-pontine transection. The hypothesis (GUALTIEROTTI *et al.*, *J. Neurophysiol.* 12: 363, 1949) that cerebellar extinction following local stimulation is due to reverberating cerebello-ponto-cerebellar circuits, i.e. brain stem inhibition, is thus disproved by these experiments.

*Interconnections between various subdivisions of the cerebellar cortex.* J. W. BARNARD AND C. N. WOOLSEY. Dept. of Physiology, Univ. of Wisconsin Med. School, Madison.

As reported earlier (*Anal. Rec.* v. 106) the paramedian lobule and the anterior lobe of the cat and monkey cerebellum are interconnected so that leg area of the one sends impulses to leg area of the other. Thus centralis and the more ventral folia of paramedian are inter-

related. Culmen, the arm area, interconnects with intermediate folia of paramedian. These connections are entirely intracerebellar. Electrical stimulation of a point in the monkey paramedian gives responses in the anterior lobe over an area covering several folia although it does not spread along the length of any one folium. Since approximately 85% of the folial surface is buried, it is computed that the response area, in some instances, is 20 times as long as it is broad. In cat cerebellar hemisphere stimulation of the medial folia of crus II causes large responses in the medial folia of crus I. Similarly the medial folia of crus I and paramedian project to the medial folia of crus II. Closer analysis reveals that the rostral parts of the folia of medial crus I connect with the caudal portions of the medial folia of crus II, the intermediate with the intermediate, and finally, the caudal parts of crus I with the rostral parts crus II. In addition a folium connects with its immediately neighboring folia although the responses are not large. The most lateral folia of the crura have only weak connections with their neighbors. Paraflocculus was examined in one instance and found to have connections with nearby folia in the adjacent crus.

*Phase-boundary potentials in nerve fibers.* T. C. BARNES AND R. BEUTNER. Depts. of Pharmacology, Hahnemann Med. College of Philadelphia, Philadelphia, Pa. and Med. College of Alabama, Birmingham.

Nerves were soaked in isotonic glucose for several hours to eliminate salts (which produce short circuits). The nerve was then placed across the air gap between two watch glasses of isotonic glucose connected with a Speedomax phase-motor recorder. By placing an electrogenic substance first in one watch glass then in the other a typical spike potential is recorded. Curves resembling spinal root potentials are produced by adding one half the dose to the second watch glass. The nerve gives higher spikes than does a thread soaked in guaiacol. It is possible to increase the phase-boundary potential of a sympathomimetic drug by treating the nerve (frog sciatic) with a triglyceride or adrenergic oil (these oils do not respond to cholinergic drugs, *Federation Proc.* 8: 272, 1949). One mg. benzedrine added to a nerve in 16-cc. glucose solution produced 38 mv. negativity but after soaking the nerve in triacetin 52 mv. were produced. These experiments suggest that the lipid in adrenergic nerves is different from that in cholinergic nerves. In oil-cell models with guaiacol in contact with physiological saline sodium chloride has the weakest electrical action of any substance tested and it is highly improbable that sodium plays any role in the action current of living nerve. Thus 30 gm. NaCl added to 200 cc. 0.9% NaCl at a guaiacol interface produced only 6 mv. negativity.

*Influence of adrenal hormones on toxic and tumor-damaging effects of certain substances.* LYLE V. BECK AND TAMARA VOLOSHIN (with the technical assistance of

MILTON PARKER). Chemotherapy Section, Natl. Cancer Institute, Bethesda, Md.

All of the toxic substances studied have the property of producing considerable gross and histologic damage to mouse sarcoma 37, when administered at MTD or lower dose levels. Mice injected intraperitoneally or subcutaneously with cortisone were found to exhibit an increased tolerance for arsenite, administered an hour later, either s.c. or i.v. Increased tolerance against both arsenite and mapharsen administered s.c. was noted for mice previously injected intraperitoneally with adrenal extract, and some protection against arsenite was secured by prior injection of ACTH. For cortisone and adrenal extract, the increase in tolerance was greater for male than for female mice, and greater at the LD<sub>5</sub> than at the LD<sub>50</sub> level. Cortisone and adrenal extract also counteracted arsenite-induced tumor damage, as judged by gross and microscopic appearance of the tumor tissue. Mice injected intraperitoneally with cortisone or adrenal extract were found to exhibit an appreciable increase in tolerance for *Serratia marcescens* tumor necrotizing polysaccharide, administered intraperitoneally an hour later. Cortisone was also found to afford protection against the polysaccharide injected i.v. Cortisone injected intraperitoneally markedly counteracted polysaccharide-induced tumor damage. There are differences between the pattern of protection against polysaccharide, on the one hand, and against the trivalent arsenicals on the other, which suggest that the protective mechanisms may differ. For example, a subcutaneous dose of cortisone which protected mice against arsenite did not protect mice against the polysaccharide. No evidence was secured for protection of mice against lethal and semilethal doses of either alpha-peltatin or certain acridines.

*Threshold of pain and skin flare of persons with malignant diseases.* F. B. BENJAMIN AND A. C. IVY. Univ. of Illinois College of Medicine, Chicago.

While examining the relationship between the threshold of pain and that of skin flare we found that the threshold for both is higher in persons with malignant disease than in a control group. This was followed by an extensive study in 1913 patients. Half of these had various neoplastic diseases and the others were normals or had other types of chronic diseases. The stimulator was an electrically heated wire applied to the inside of the forearm. A definite and consistent relationship between the threshold of pain and that of skin flare was obtained only in those specifically trained to perceive the first appearance of pain sensation. In all others considerable variability occurred in the determination of a threshold for pain. But the threshold of skin flare was definite and consistent, and it showed a very striking and significant difference between the cancer and the non-cancer bearing subjects. In the neoplastic group only patients with brain tumors and those with Hodgkin's Disease gave no consistent response, while those

with benign tumors reacted like normals. The administration of steroid sex hormones to patients with cancer of the breast or prostate caused the threshold of flare to fall for a prolonged period. Intensive x-ray therapy did the same but only for a period of a few hours.

*Modification of the methods for determination of inulin space.* ROBERT M. BERNE (introduced by CARL J. WIGGERS). Dept. of Physiology, Western Reserve Univ. Med. School, Cleveland, Ohio.

Inulin space determination in the dog, using the continuous infusion technique as modified by Gaudino and Levitt, involves the collection of urine for 5 hours in order to recover the inulin remaining in the dog at the moment the infusion is stopped. We have found, however, that at the end of the 5-hour collection period 5.8% of the total amount of inulin given was not recovered, and 3.6% was still missing at the end of eight to 11½ hours. In terms of the inulin remaining in the dog, when the infusion was discontinued, this is equivalent to a 17% and 9% error, respectively. Earlier methods consisted of subtracting the amount of inulin excreted during the infusion (a large figure) from the total amount given to obtain the number of milligrams in the dog (a small figure). This has been criticized because in the analyses of these larger fractions the experimental error may be additive and yield a much greater per cent error (14.6%) of the smaller fraction. This error can be reduced by accurately weighing out the inulin to be given and dissolving it in a known volume of saline. Analysis for the inulin excreted during the infusion is accurate to within 1.5%, using the method of Higashi and Peters. Therefore, it appears that the older method has a smaller error, does not require extended periods of time for urine collection, and the error attending analysis of urine with very low inulin concentrations is avoided.

*Effect of posture on cardiac output in man.* M. M. BEST,\* W. S. COE\* AND H. C. LAWSON. Univ. of Louisville Institute for Med. Research, Louisville, Ky.

Reports in the literature on the effects of posture on cardiac output are controversial. Cardiac output, as influenced by postural changes, was studied by the intracardiac dye injection method (see Coe *et al.*, this issue) in human subjects while on a tilt table. The 30° head-up posture caused a decrease in cardiac output in 19 of 20 determinations. The average decrease was 19.2 per cent. The 15° head-down posture had an inconstant effect on cardiac output. Ten of 18 patients had an increase in cardiac output. The average increase in all patients was 1.8 per cent. Deep sedation and curare had no significant effect on cardiac output in the various tilt-table positions. The posture yielding the greatest increase in cardiac output was one in which the subject was supine with the legs extended in a vertical position.

*Relationship between p-aminohippurate synthesis and phenol red secretion by the mammalian kidney.* KARL

H. BEYER, VIRGIL D. WIEBELHAUS, KATHARINE M. WILHOYTE AND ROBERT L. KEMP.\* Dept. of Pharmacology, Med. Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

Previous reports indicate that the conjugation of p-aminobenzoate (PAB) with glycine to form p-aminohippurate (PAH) (BEYER, WIEBELHAUS, TILLSON, RUSSO AND WILHOYTE: *Proc. Soc. Exper. Biol. & Med.*, in press), and the renal tubular transport system for the secretion of phenol red and PAH (BEYER, PAINTER AND WIEBELHAUS: *Am. J. Physiol.* 161: 259, 1950) are 'coupled' systems that require the integrity of high-energy phosphate mechanisms in order to complete the conjugative or secretory process. Present evidence indicates a parallelism between the two systems of glycine conjugation and phenol red or PAH secretion sufficient to suggest that they are causally related. The inhibitory effects of 'Benemid,' Carinamide and some of their analogs, certain cinchoninic acid and ethylene-diamine derivatives, and a number of other common enzyme inhibitors on the two systems have been studied by the procedures described in the above references. The results indicate that: 1) In general there is a parallelism between the inhibitory effects of a given compound for the two systems. 2) This correlation holds for both 'specific' inhibitors of the conjugase or secretory systems, such as 'Benemid' and Carinamide, and the 'nonspecific' inhibitors of the phosphorylative or oxidative components of the system, such as dinitrophenol, cyanide or phloridzin. 3) Among a systematic series of compounds belonging to a single class there is a semiquantitative correlation of their inhibitory effects on the two systems, within the limits of variability of absorption of the compounds by the renal tubular cells and the destruction of the compounds by the extraneous systems contained in the material used for the conjugase studies.

*Method for approximation of residual volume of blood in right ventricle.* R. J. BING, R. O. HEIMBECKER, W. FALHOLT, A. FRIEDLICH AND D. CARROLL. Johns Hopkins Hospital, Dept. of Surgery, Baltimore, Md.

The construction of a photoelectric constant dye recorder has simplified the determination of cardiac output with Evans Blue. This recorder has now been employed in the estimation of the residual volume of blood in the right ventricle of man. A double lumen catheter was introduced in such a manner that the opening of one twin catheter was in the pulmonary artery, while 5 openings of the other twin catheter were placed in the right ventricle. Evans Blue was then injected through the ventricular openings, and blood was withdrawn at a constant rate from the pulmonary artery through the cuvette of the dye recorder. The photoelectrically recorded curve was then plotted on semilogarithmic paper. The dye dilution curve was found to be a straight line. There was evidence of satisfactory mixing of the dye with right ventricular blood.

The factors which influenced the slope of the dye curve were found to be the cardiac output, the right ventricular residual volume and the conducting system from pulmonary artery to the cuvette of the recorder. Since the cardiac output could be determined, the residual volume could be calculated from the slope, provided the influence of the conducting system could be ascertained. To estimate this, an artificial system was employed in which residual volumes and flows could be varied independently. From this a factor for the conducting system could be obtained. After correcting for this factor a linear relationship was found to exist on semilogarithmic paper between the ratio flow/residual volume and the slope of the curve. The residual volume of the right ventricle could then be obtained from this relationship. The residual volume of normal right ventricle varied from 60 to 120 cc. Larger volumes were found in failure.

*Semi-automatic computing equipment for determination of nerve membrane admittance characteristics.* Biophysics Group (introduced by OTTO H. SCHMITT). Univ. of Minnesota, Minneapolis.

The escape of stimulus along a nerve into the regions extrapolate to the stimulating electrodes is usually considered merely a nuisance. It can be shown, however, that for subthreshold sinusoidal stimulation, the rate of decay of this potential and the accompanying phase-shift determine uniquely the conductance and susceptance of the nerve membrane if the dimensions of the nerve and its longitudinal resistance are known. Instrumentation to record and evaluate this type of data quickly, accurately and semi-automatically has been developed and will be demonstrated. A main computer unit records simultaneously on two sheets of graph paper the logarithmic amplitude and the phase of signal against position along the nerve for any chosen stimulation frequency. The resultant curves are straight lines for any uniform nerve and thus provide an immediate inspection test for the uniformity of a nerve preparation. A second instrument, essentially a mechanized two dimensional nomogram, accepts the graph sheets from the computer and reads from them direct numerical values for the conductance and susceptance of the nerve membrane. A specially designed thermostated nerve chamber and other auxiliary equipment used in this work are also shown.

*Treatment and prophylaxis of experimental renal hypertension with semi-purified hog renin.* R. B. BIRD,\* BESS OSGOOD,\* R. W. SEVY\* AND G. E. WAKERLIN. Dept. of Physiology, Univ. of Illinois College of Medicine, Chicago.

Four chronic hypertensive dogs were injected intramuscularly and daily for 6 months with 2.5 DU (Goldblatt) per kg. of semi-purified hog renin (70 DU/mg. N), prepared by ethanol fractionation of desiccated, defatted hog renal cortical tissue. Reductions in blood

pressure ranged from one-third of the way toward normotension to normotension. The antihypertensive responses were excellently correlated with the antirenin titers. Four normotensive dogs were similarly injected with semi-purified hog renin for 4 months prior to successive bilateral renal artery constriction. Three of these dogs were protected against the development of hypertension. The minimum effective antirenin titer for protection was 2.0-3.0 DAU (Dog Antirenin Units) per cc. serum. One dog with a titer of 1.0 DAU was not protected. The semi-purified hog renin used was prepared from hog renal cortical tissue *completely free of medulla* and extracted with saline-bicarbonate solution for 16 to 20 hours. These antihypertensive results are superior to those reported previously with semi-purified hog renin, due to complete absence of the interfering action of medullary protein. The reduced susceptibility to uremia observed in the normotensive dogs whose renal arteries were constricted following prophylactic courses of semi-purified hog renin is attributable to shorter extraction of the renal cortical tissue. Antirenin has been conclusively demonstrated to be responsible for the antihypertensive effects of semi-purified hog renin. These findings, together with others previously and presently reported by our research group, are excellent evidence in support of the renin-hypertensin mechanism in the pathogenesis of early and chronic experimental renal hypertension.

*Carbon dioxide asphyxiation of some British Columbia fresh-water fishes.* EDGAR C. BLACK AND VIRGINIA S. BLACK.\* Dept. of Physiology, Faculty of Medicine, Univ. of British Columbia, and the British Columbia Game Department, Vancouver.

Ten species of fish acclimated to 11° C. were asphyxiated at this temperature by sealing individual fish in bottles of water containing various tensions of dissolved carbon dioxide and adequate oxygen. The ambient water was analyzed for dissolved oxygen and free carbon dioxide when respiratory movements had stopped. Curves were constructed from these data. The levels at which carbon dioxide prevented utilization of oxygen at an arbitrary level of oxygen tension of 160 mm. Hg were as follows: Kokanee, 84 mm.; Kamloops trout, 109 mm.; Prickly sculpin, 114 mm.; Red-side shiner, 126 mm.; Pumpkinseed, 126 mm.; Yellow perch, 136 mm.; Long-nose dace, 148 mm.; Fine-scale sucker, 168 mm.; Carp, 237 mm.; Chub, 254 mm. The use of these data in describing certain ecological relationships of these fishes is discussed. (This study was supported by a grant from the Natl. Research Council, Ottawa, Canada.)

*Prepyloric deviation of alkaline secretions in relation to histamine ulcer.* G. C. BLANCHARD (introduced by W. F. HAMILTON). Dept. of Surgery, Med. College of Georgia, Augusta.

A control group of 5 normal dogs were given daily

intramuscular injections of a histamine-in-beeswax mixture. They all died of ulcers in periods of 4 to 30 days. In a second group the pancreatic juice was deviated into the stomach antrum. This was accomplished by a variety of surgical procedures and the same dose of the histamine mixture was administered daily. Those dogs in which the anastomosis between pancreas and stomach did not function proved very susceptible to ulcer formation and died in 2 to 8 days. Of 10 dogs with functioning anastomoses, 6 died of ulcer in periods of 15 to 56 days, but 4 showed no pathology or only erosions when killed after 40 to 90 days. In a third group of 8 dogs the bile was deviated into the antrum by common duct ligation and cholecystgastrostomy. They proved quite resistant to the histamine ulcer, and all showed no pathology or only erosions or duodenitis when they were killed after 40 to 90 days on the histamine mixture. Gastric analyses in some of the animals revealed that acidity might be high or low in the absence of ulceration. The drug 'Banthine' was employed in 2 normal dogs in doses of 5 mg./kg. 3 times daily with concomitant injections of the histamine-in-wax mixture. They died of ulcers after 5 and 8 days. Two other dogs received the preparation 'Cortone' in daily doses of 20 mg. concomitantly with the histamine mixture. They lived for 21 days at which time cortone was discontinued. They subsequently died of ulcer 15 and 21 days after the cortone had been stopped.

*Regeneration of visual purple in solution.* ALFRED F.

BLISS. Dept. of Physiology and Biophysics, Georgetown Univ. School of Medicine, Washington, D. C.

Visual purple bleaches in light with the release of vitamin A aldehyde (retinene), which then is reduced to vitamin A alcohol. We have shown that the reduction is reversible (*Biol. Bull.* 97: 221, 1949), both in the retinal rods and in liver extracts containing alcohol dehydrogenase. The degree of reversibility is remarkably high, the equilibrium constant for the dehydrogenation being about 300 times that of ethyl alcohol. When solutions of bleached visual purple, containing vitamin A aldehyde, are placed in the dark, visual purple is regenerated. A factor from the melanotic pigment layer of the eye, which may be a protein, is required for normal regeneration. In addition, it has been reported (CHASE AND SMITH, *J. Gen. Physiol.* 1939) that an accessory blue-sensitive substance is involved in regeneration, since a much greater increase in optical density in the dark was found after bleaching visual purple with blue light than after orange light. However, we have been unable to demonstrate this effect in extracts of visual purple reinforced with pigment layer extracts, using as a criterion of regeneration, the photosensitive optical density at 500 mμ. The effect obtained by the earlier investigators may have been due to bleaching by blue light of unstable orange intermediates (SEGAL, *Comp. rend.* 144: 403, 1950). After orange light, a slow fall in density in the dark of the unbleached

intermediates might have masked the increase in density due to regeneration of visual purple.

*Studies in iodine utilization. I. Role of the erythrocyte in blood iodine transport using radioiodine  $I^{131}$ .*

JOSEPH B. BOATMAN, THOMAS R. KENDRICK AND THOMAS F. NEWCOMB. Addison H. Gibson Lab., Univ. of Pittsburgh School of Medicine, Pittsburgh, Pa.

Six groups of 10 albino rats were sacrificed at time intervals of 15 minutes to 24 hours after receiving 200 microcuries of  $I^{131}$  intraperitoneally. The amount of radioiodine in the whole blood and plasma and erythrocytes was determined. Determinations of the amount of radioiodine in the circulating whole blood volume, the plasma and the erythrocyte fractions were made for each period. The radioactivity of the erythrocyte was determined subtracting the value of the plasma from the whole blood assay, after corrections were made for hematocrit ratios. These determinations were found to be approximately 5% below the direct assay of the packed centrifuged erythrocytes. The whole blood percentage activity of the injected  $I^{131}$  declined from 19.6% at 15 minutes to 0.46% at 24 hours. The plasma declined from 12.52% at 15 minutes to 0.34% at 24 hours, and the erythrocytes from 6.15% at 15 minutes to 0.12% at 24 hours. This loss from the blood was considered to represent the thyroid uptake, kidney excretion and the dispersion into the body fluid compartments. The percentage of whole blood radioactivity retained by the erythrocytes was found to be nearly constant over the 24-hour period, from 36.2% of the whole blood radioiodine at 15 minutes to 35.9% at 24 hours, indicating that loss of radioiodine from the whole blood was quickly adjusted between the erythrocyte and the plasma. When the percentage of whole blood of radioiodine in the erythrocyte was compared with the estimated percentage of whole blood water in the erythrocyte, the ratio was nearly equal for the first 2 to 6 hours, and declined at 24 hours to 0.87. It was concluded the data support the view that the erythrocyte iodine content is a function of the erythrocyte water content.

*Comparison of influence of ACE, DCA and cortisone on metabolism of ascorbic acid.* WALTER M. BOOKER, FRANCES M. DENT, RAYMOND L. HAYES, WARREN HARRIS AND SOLOMON GREENE. Depts. of Pharmacology and Oral Medicine, Howard Univ. Colleges of Medicine and Dentistry, Washington, D. C.

The following experiments were performed: 1) A comparison of the 24-hour excretion of vitamin C in control dogs, in dogs given vitamin C daily, and in dogs given vitamin C along with a) ACE, b) DCA and c) cortisone. 2) A comparison of the plasma and blood cell levels of vitamin C from day-to-day in control dogs, in dogs given vitamin C, and in dogs given vitamin C and one of the above adreno-steroids. 3)

The 24-hour urinary excretion of vitamin C in rats under influence of the adreno-steroids, and the plasma and cell levels at sacrifice. 4) Tissue distribution of vitamin C in rats given vitamin C and one of the above adreno-steroids. Our results show that in dogs given DCA daily along with vitamin C, the urinary excretion of vitamin C is slightly less than that of cortisone-vitamin C, which is about equal to that of ACE-vitamin C. The adreno-steroids as a group exert roughly 60 to 75% reduction of vitamin C, as compared with the vitamin C treated animals alone (dogs and rats). The adreno-steroids as a group maintain higher day-to-day plasma and cell vitamin C levels with no great difference between ACE, DCA and cortisone. The cell and plasma levels of the latter are only slightly higher than the first two (dogs). In rats, at sacrifice, the same general picture is seen in the blood ascorbic acid, with cortisone, DCA, and ACE (in this order) being outstanding in high levels of vitamin C particularly in blood cells. In the matter of tissue distribution, the adreno-steroids exert their greatest influence on storage of ascorbic acid at the adrenal gland in the order of ACE, cortisone and DCA, above the vitamin C or untreated animals. ACE, cortisone and DCA increase the storage of vitamin C at lung. Muscle vitamin C storage is increased markedly by DCA.

*Equilin, an orally active estrogen in women.* JAMES T. BRADBURY AND ROBERT C. LONG. Univ. of Louisville, Louisville, Ky.

In 1932 Girard reported the isolation of a new natural estrogen, equilin, from pregnant mare's urine extracts. In 1935 several investigators tested equilin for its estrogenic potency and found it as active as, or less active than estrone in rats and mice. The lack of a high estrogenic potency discouraged further investigation and equilin was apparently never given a clinical trial. Vaginal smear studies in postmenopausal women indicate that a daily oral dose of 0.5 mg. of equilin gives a response comparable to that obtained with 1 mg. of estrone daily. Biopsy studies in menopausal women show that 1 to 2 mg. of equilin/day will restore the atrophic endometrium to a normal proliferative phase. Ryden, 1950, reports that 20 to 24 mg. of oral estrone is necessary to duplicate this response. Withdrawal bleeding is induced in women after daily doses of one mg. of equilin for 10 days and 2 mg. daily for the next 10 days. Equilin, one mg. daily, plus 80 mg. of Pranone led to early secretory changes in the endometrium in one week. Equilin, 2 mg. daily, together with 100 mg. of Pranone gave early secretory changes in one week and good decidual changes in 2 weeks. Thus equilin is an effective estrogen for augmenting progestational changes in the endometrium. The high urinary gonadotrophin excretion of the postmenopausal patient is promptly reduced by equilin in doses of one mg/day. These observations indicate that equilin

is worthy of further study as an orally active natural estrogen.

*Treatment of experimental renal hypertension with antirenin.* T. L. BRANNICK\*, B. G. OSGOOD\*, R. O. BURNS\* AND G. E. WAKERLIN. Dept. of Physiology, Univ. of Illinois College of Medicine, Chicago.

Our research group has now demonstrated that the efficacy of crude and semi-purified hog renins in the therapy and prophylaxis of experimental renal hypertension in the dog is due to antirenin. Inconsistent and inconclusive results in the past with crude and semi-purified hog renins have been due mainly to contamination with hog renal medulla protein. Accordingly, reductions in blood pressure should be possible by passive treatment with antirenin. In preliminary experiments, antihypertensive effects with antirenin to semi-purified hog renin produced in the dog were observed on administration to renal hypertensive dogs. No effect was seen in two experiments where the antirenin titers reached only 2.5 and 3.0 DAU per cc. of serum. In the remaining 4 experiments, antihypertensive effects were seen, particularly in two dogs with antirenin titers of 5.0 and 7.0 DAU whose blood pressures were reduced to approximate normotension. Effects were obtained with antirenin in the form of antiserum and partially purified antirenin, subcutaneously as well as intravenously. The critical factor appeared to be the antirenin titer, passively produced, strongly supporting renin as the pathogenetic mechanism of experimental renal hypertension. Further work with concentrated purified antirenin is necessary. Since antirenin to hog renin does not neutralize human renin, similar experiments on the effect of antirenin to human renin on human hypertension are planned to clarify the possible renal pathogenesis of essential hypertension.

*Action of desoxycorticosterone on water exchange in the rat.* EDUARDO BRAUN-MENENDEZ (introduced by ERIC OGDEN). Institute of Biology and Exptl. Medicine, Buenos Aires, Argentina.

We have recently shown that in the absence of dietary salt the chronic administration of desoxycorticosterone acetate (DCA) does not cause hypertension, or renal lesions, or changes in the water intake or diuresis (BRAUN-MENENDEZ AND PRADO, 1950). Our present experiments show that subcutaneous injection of 2.5 mg DCA per rat per day for 10 days caused no increase in water intake in rats fed a salt poor or salt rich diet. But, when the only drinking fluid available is a 0.1% solution of NaCl then a marked increase in fluid intake and in diuresis occurred and if the rats were allowed a free choice between saline solution and water they increased the intake of the former. Rats given access to a 0.1% NaCl solution in a container separate from the one used for the drinking tap water were injected with DCA (2.5 mg/day). The second

day after initiating the administration of the steroid the rats began to increase markedly their intake of NaCl solution while water intake decreased or did not change. The increased appetite for the saline solution persisted until 2 to 3 days after the injection period of 10 days. In another experiment rats were allowed free choice between various salt solutions. To one group 0.17 M solutions of NaCl, KCl,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{NH}_4\text{Cl}$  and water were offered; to the other, 0.17 M solutions of NaCl,  $\text{Na}_2\text{HPO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaHCO}_3$ , Na citrate and water. The administration of DCA (2.5 mg/rat/day) during 10 days caused in the former group a marked increase in intake of NaCl solution (from 5 ml/100 gm/day in the control periods to 11.5 ml/100 gm/day). A small increase in the intake of  $\text{NH}_4\text{Cl}$  and a decrease in  $\text{MgCl}_2$  and water intake also occurred. In the second group the intake of both NaCl and  $\text{Na}_2\text{HPO}_4$  increased significantly, and as the injection period progressed the rats seemed to drink more NaCl solution in preference to  $\text{Na}_2\text{HPO}_4$ . As in the former group water intake was reduced during the DCA administration period. Subcutaneous administration of DCA to normal rats (2.5 mg/rat/day during 10 days) caused a very marked increase in their intake of NaCl solution even if water or other saline solutions are available. These results cannot be explained simply by an augmentation of thirst due to the sodium retaining action of DCA.

*Effect of pulmonary stenosis upon the circulation in the absence and presence of an interatrial septal defect.*

G. A. BRECHER AND D. F. OPDYKE. Dept. of Physiology, Western Reserve Univ. Med. School, Cleveland, Ohio.

Left and right atrial pressure relations were investigated in dog experiments during right ventricular failure, produced by acute progressive pulmonary artery occlusion before and after production of an interatrial septal defect. Acute pulmonary stenosis reduced left and increased right atrial pressures in normal animals, resulting in a reversal of the usual left to right interatrial pressure gradient. Left atrial pulse contours became almost obliterated, whereas right became more pronounced. Left atrial inflow during ventricular systole decreased, as indicated by the declination of the gradient between the Z and V point (Z-V gradient). Right atrial 'inflow' (venous return plus tricuspid regurgitation) increased simultaneously, as concluded from the inclination of the right Z-V gradient. Right heart failure, characterized by right myocardial overdistension with decreasing right intraventricular systolic pressures, developed at the same degree of pulmonary stenosis, which also caused right atrial pressure to supersede left. In the presence of a small interatrial septal defect late atrial pressures decreased only slightly and right increased less than normally. Acting as a 'safety valve,' a small defect alleviated right heart failure (myocardial overdistension) and venous congestion by shunting some of the right atrial blood

to the left atrium. In the presence of a large defect left atrial pressure rose also when right pressure increased. In such cases aortic pressures were maintained even during complete pulmonary occlusion, indicating a transfer of large amounts of blood through the shunt from the right to left atrium.

*Radiomanganese studies in the pancreatic fistula dog.*

W. T. BURNETT, JR., R. R. BIGELOW AND C. W. SHEPPARD. Biology Div., Oak Ridge Natl. Lab., Oak Ridge, Tenn.

Radiomanganese appeared in the pancreatic juice of dogs provided with pancreatic fistulae within a half hour after intravenous administration. Stimulation of the pancreas by feeding increased the rate of appearance, which was found to parallel that of protein nitrogen, thus suggesting that the secretion of manganese may be a normal function of the acinar cells. The congruence in the secretion patterns of manganese and protein points to an intimate relationship between the micronutrient and the pancreatic enzyme systems. A comparison of recovery values for radiozinc in pancreatic juice reported by Montgomery *et al.*, with those for radiomanganese raises the question as to a common, or perhaps interchangeable, metabolic role of zinc and manganese in the pancreas, a gland which, as shown by a few scattered data in the literature, has an affinity for both elements. Two of the dogs were each given 7 mg. of Mn as the chloride, labeled with 54 Mn; 555  $\mu$ c. in one case (*dog 1*) and 333  $\mu$ c. in the other (*dog 2*). *Dog 1* secreted 0.62% of the dose in 315 ml. of pancreatic juice during the first 24-hour interval and 0.42% in 599 ml. during the following interval. *Dog 2* secreted 0.05% in both 55 and 94 ml. during the first and second intervals, respectively. The total secretion of *dog 2* over a 13-day period was 0.38% in 1099 ml. Very low concentrations of radiomanganese in the plasma appears to exclude the possibility that there was an ultrafiltration of manganese from the plasma to the pancreatic juice.

*Cineradiographic studies on circulatory effects of hemorrhage and intravenous infusions.* JOHN L. CANNON,\*

HAROLD L. MURRAY,\* HEINZ S. WEENS,\* AND JAMES V. WARREN. Depts. of Physiology and Radiology, Emory Univ. School of Medicine, Atlanta, Ga.

Utilizing the technique described in the previous paper, some 45 cineradiographic observations—control, following hemorrhage, and following infusion—were made. Diodrast was injected following each procedure, permitting visualization of all chambers of the heart. The resulting angiocardigram was analyzed frame by frame; frames which represented maximum systole and maximum diastole were subjected to special analysis. Each of these selected frames was projected and the silhouette of the heart chambers traced. Their projected areas were then measured with a planimeter. The change in the area from diastole to

systole of either ventricle then represented 'stroke area.' The 'stroke area' multiplied by the heart rate gave 'square inches per minute' output. Such figures could then be compared after hemorrhage and infusion, but only in the same animal. Following hemorrhage the diastolic and systolic ventricular areas decreased, diastolic more than systolic, so that in square inches per minute output decreased. Following infusion, there occurred an increase in diastolic and systolic areas with the square inches per minute output being increased. It was also found that the atrial change in area during each cardiac cycle was striking. A high degree of correlation existed between the change in atrial area and the change in ventricular area during the same cardiac cycle. Such correlation suggests that the atrium plays a large role in ventricular filling.

*Contrast visualization of the cardiovascular system utilizing a method of slow motion cineradiography.* JOHN L. CANNON,\* JAMES V. WARREN AND HEINZ S. WEENS.\*

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A method, first suggested by Rehman, for taking 60 pictures/second was considerably modified and improved so that actual photography was completely automatic. One-half wave rectified current was supplied to the x-ray tube which responded with an output of exactly 60 bursts of x-ray beam/second. A 35-mm. camera (equipped with an f/0.9 lens) was driven at exactly 60 frames/second by a synchronous motor. Since the motor alone was unable to determine when the peak of screen fluorescence occurred, a method of changing the shutter opening so that it was always over the lens aperture at the proper time was needed. A unit for automatic adjustment was devised utilizing a mechanical rectifier, an electronic rectifier and a solenoid coil which activated a differential gear train to change the camera shutter-motor shaft relationship when necessary. By starting the motor the synchronizer unit determined whether or not the camera shutter was open at the proper time and if not, the change was made by energizing the solenoid coil automatically, which, in turn, activated the differential gear. Very satisfactory cineangiocardigrams were obtained in animal (canine) subjects. A second electronic device was developed which permitted cineradiographic events to be accurately correlated with other biological measurements, such as electrocardiograms, heart sounds and various blood pressures. This device automatically marked each thirtieth frame of the film and simultaneously placed a marker on a 4-channel, direct-writing recorder.

*A comparison of protective action of the dihydrogenated ergot alkaloids to cyclopropane-epinephrine ventricular tachycardia.* ROBERT T. CAPPS,\* HENRY M. SUCKLE\*

AND O. SIDNEY ORTH. Dept. of Pharmacology, Univ. of Wisconsin Med. School, Madison.

Four dihydrogenated alkaloids of ergot, Dihydroergotamine (DHE 45), Dihydroergocornine (DHO 180), Dihydroergocristine (DCS 90) and Dihydroergokryptine (DHK 135), each as the methanesulfonate salt, (Sandoz Pharmaceuticals), have been tested for their ability to block ventricular cardiac arrhythmias produced with epinephrine during cyclopropane anesthesia. The stimuli initiating the cardiac irregularities are believed to pass via thoraco-lumbar autonomic nerve fibers, and the effect of the drugs in interrupting or blocking the passage of such stimuli was taken as the basis for comparison of the alkaloids. Dogs were anesthetized with mixtures of cyclopropane and oxygen and then maintained at the level of mid-third plane surgical anesthesia using a to-and-fro absorption system with approximately 30% cyclopropane in oxygen. The doses of epinephrine and the dihydrogenated ergot alkaloids were diluted to a standard 5-cc. volume and injected i.v. at a constant rate during definite intervals. Electrocardiograms were made at regular intervals during and following the injections of the drugs and at other intervals when cardiac irregularities were present or were expected to occur. The composite data indicate that DSC 90 is the most effective of the 4 compounds in preventing epinephrine-induced ventricular tachycardia. DHO 180 was found to be least effective and DHE 45 and DHK 135 intermediate in this respect. Comparison of the 4 alkaloids in the same dogs, however, at doses of 0.4 mg/kg. and 0.2 mg/kg., did not establish a specific advantage for any one of the alkaloids on the basis of either degree or total duration of protection.

*Effect of segregation and isolation before mating on the fertility of rats.* ANTON J. CARLSON AND FREDERICK HOELZEL. Dept. of Physiology, Univ. of Chicago, Chicago, Ill.

One hundred male and 131 female offspring of Wistar rats raised during 3 or more generations on a rich, a bulky or a relatively poor diet (*Federation Proc.* 9:354, 1950) were used. Approximately equal numbers were raised on the parental diets after weaning either in segregated groups or in separate cages (isolation). Littermates were mated when they became between 127 and 229 days old. The rats were regarded as sterile when no pregnancy resulted in 30-day mating tests with rats found to be fertile during or before and after the tests. The highest percentage of sterility (males 76%, females 65%) was found in the isolation group on the rich diet and the lowest sterility (males 19%, females 27%) in the segregation group on the relatively poor diet. The percentage of sterility and impaired fertility was practically proportional to the weights attained. The average weight of the rats in all isolation groups was greater than the average weight of the rats in the corresponding segregation groups and the rats on the rich diet were heaviest. The isolated rats evidently became heavier than the segregated rats

because of less activity and less distraction from eating. The results correspond with the estimate of 16% sterility in the human leisure classes and 10% in the working classes (S. R. MEAKER, *Human Sterility*. Baltimore: Williams & Wilkins, 1934).

*A convenient and adaptable thermostated nerve chamber.*

KENT M. CHAPMAN AND MARGARET WATKINS (introduced by OTTO H. SCHMITT). Biophysics Group, Univ. of Minnesota, Minneapolis.

A nerve chamber has been built from a copper block in which temperature regulation is achieved by a battery of heating lamps and a system of cold water circulating tubes controlled by a thermistor imbedded in the block. The chamber is hermetically sealed by the use of 'O' rings, but permits rapid changing of the environment by means of hypodermic stopcocks. The temperature of the chamber is read by a thermistor thermometer. A thick plexiglass cover provides good heat insulation and allows full visibility. Temperature equilibration of the environment is maintained by a small stirring propeller. Nerve preparations are mounted by threads which are clamped into adjustable, tightly wound coil spring clamps.

*An alteration in visual function resulting from unilateral frontal lesions in the rat.* GEORGE CLARK AND S. E. GOLDBERG.\* Dept. of Anatomy, Chicago Med. School, Chicago, Ill.

There are numerous reports in the literature of transient visual disturbances following unilateral frontal lesions. While the location of the lesions responsible and the nature of the defects have been described, there has been as yet no elucidation of the neural processes involved. This preliminary study was begun in the hope that if similar disturbances could be demonstrated in the rat it would be possible to determine the anatomical substrate. Using a Y maze for testing it was found that the animals will run only to the side of the lesion for a transient period following amputation of the lateral  $\frac{1}{4}$  of the anterior portion of the cerebral hemisphere. However more than visual factors are concerned so the rat proved to be unsuitable for further study of this phenomenon.

*Leukocytosis in the rat upon oral administration of a bacterial pyrogen.* CARMINE D. CLEMENTE (introduced by W. F. WINDLE). Dept. of Anatomy, Univ. of Pennsylvania, Philadelphia.

Upon oral administration of a bacterial pyrogen, Pyromen, at dosages of one gamma/rat/day, a significant leukocytosis was observed. This occurred after one to 4  $\mu$ g. of Pyromen had been administered in a constant dilution. The white blood cell counts increased from a normal of 15,000 cells/cu. mm. to 35,000 or 40,000 cells/cu. mm. After sustained dosages of a constant dilution, a tolerance effect was noted at which time white blood cell counts returned to normal. Increasing the dosage while the animal was returning



to a normal blood picture appeared to have no effect. After a return to normal, increased dosages had a secondary leukocytic reaction only if the animal had been normal for about 6 days. Secondary leukocytosis did not seem to be nearly as marked nor as prolonged. The percentages of granular to non-granular leukocytes did not seem to change even in the greatest periods of leukocytosis. A slight leukopenia was observed soon after commencement of dosage in 2 of the 4 experimental animals. Eosinophils seemed to behave like all other granulocytic cells. In 2 cases there was an early decrease in circulating eosinophils. During leukocytosis, eosinophils increased in proportion to other granular cells. No consistent gross or microscopic pathology due to oral administration of Pyromen was evident in any of the endocrine, digestive, lymphatic or urinary organs. (Aided by a grant from Baxter Laboratories, Inc., Morton Grove, Ill.)

*Measurement of cardiac output by intracardiac dye injection.* W. S. COE,\* M. M. BEST,\* AND H. C. LAWSON. Univ. of Louisville Institute for Med. Research, Louisville, Ky.

Conventional cardiac catheters were used for injecting solutions of T-1824, concentration 2.5 to 5 mg/cc, in volumes of 2 to 3 cc, into the right heart and adjacent great vessels. Each catheter, along with a standardized syringe, was calibrated for constancy of delivery under extreme deformation and with agitation of the tip. Delivery was found to be constant within 3 per cent. Injections were accordingly made without flushing the catheter, the syringe remaining undisturbed until blood sampling was complete. Peripheral vein injections, in similar volumes, were done by needle puncture. Methods for arterial sampling, plotting of time-concentration curves, and calculation of outputs were essentially those described by Hamilton and colleagues. Data have been obtained with the following injection sites in man: antecubital vein at heart level, antecubital vein elevated, superior vena cava, right atrium, right ventricle and pulmonary artery. The two peripheral injections yield curves which are greatly elongated along the time axis, shortening of the time interval between comparable points on the curves, with the central injections, amounting to as much as 50 per cent. Cardiac output has been calculated from paired injections, a peripheral vein at heart level and the right ventricle, in a series of barbitalized dogs and a series of normal humans. In both series the ventricular injection usually yielded the larger value for output, suggesting the presence of an error due to undetected recirculation of dye, when the dye is injected in a peripheral vein under these conditions.

*Effect of dietary variations on response of liver slices from hyperthyroid rats to various substrates.* FRANCES M. COLVIN AND SAMUEL R. TIPTON. Dept. of Zool-

ogy and Entomology, Univ. of Tennessee, Knoxville.

The administration of desiccated thyroid powder to albino rats results in an increase of 30% in the  $Q_{O_2}$  of liver slices. If the animals have been maintained on a riboflavin-deficient diet when the thyroid is given the increase in  $Q_{O_2}$  is about the same as that of hyperthyroid rats on a normal diet; however, if they are thiamin-deficient the liver slices show no significant change after thyroid is given. The liver slice  $Q_{O_2}$  of control rats is increased on addition of alanate, glutamate, pyruvate and succinate. After thyroid administration the percentage increase with these substrates is about the same, or even less, than it is in liver slices from rats on normal thyroid. Where liver slices from control rats in a hyperthyroid condition show less increase after succinate is added than do slices of normal rats, those from thiamin-deficient hyperthyroid and riboflavin-deficient hyperthyroid animals show a significantly greater percentage increase after succinate addition than do slices from vitamin-deficient rats in a normal thyroid condition. The vitamin deficiency may depress some enzyme system which is competing with succinoxidase for available substrate, and which is affected by thyroid hormone.

*Circulatory reflexes in the rat when tipped to the head-up position.* RUTH E. CONKLIN AND FLORENCE THEOPHILUS. Vassar College, Poughkeepsie, N. Y.

In view of the increasing use of the rat for circulatory studies, experiments were planned to test their ability to adjust to the upright position, before and after elimination of the receptors of the carotid and aortic regions. Blood pressure was recorded with a double membrane manometer from the carotid artery. Tipping to the head-up position always produces a drop in blood pressure, but there is often a compensatory rise during the tipping period. In the normal condition the females showed a slightly greater fall in blood pressure than the males, and less compensation. However, after double vagotomy and later temporary obstruction of the second carotid artery, the females showed less increase in blood pressure than the males as a result of elimination of moderator impulses, less fall with change in posture, and slightly more compensation. In this respect the female rat seems to resemble the rabbit, whereas the male rat gives a response more like that obtained by Mayerson in the dog. These differences seem to indicate that even though the female has a more flabby abdominal wall, it has a slightly more stable circulatory system than the male, enabling it to make a somewhat better adjustment to adverse circumstances.

*Effects of autonomic agents on renal water, sodium and potassium clearance.* DONALD L. COOK, W. E. HAMBOURGER AND D. M. GREEN. G. D. Searle and Co., Chicago, Ill.

The effects of various autonomic agents on the renal clearances of water, sodium and potassium were studied in a group of dogs anesthetized with sodium pentobarbital. Urine was collected by cannulation of the two ureters. Control clearance values were established during two or more 15-minute periods. The agents primarily studied were acetylcholine, adrenalin and arterenol. They were administered by constant infusion for 15-minute periods through a catheter opening into the aorta just above the renal arteries. Their effects were measured before and after the administration of blocking agents, including atropine, tetraethylammonium bromide, Banthine bromide and Dibenamine hydrochloride. Acetylcholine in doses ranging from 1 to 16  $\mu\text{g/kg/minute}$  increased water and electrolyte clearances by 50 to 300 per cent without significant change in blood pressure. Larger doses or more rapid rates of administration were associated with blood pressure depression and slight to moderate reduction in renal clearances. These effects were more or less completely prevented by prior administration of atropine or Banthine, but were not inhibited by continuous administration of tetraethylammonium bromide. Adrenalin in doses greater than 1  $\mu\text{g/kg/minute}$  caused marked reduction in water and electrolyte clearances and increased systemic blood pressure. Doses of 1  $\mu\text{g/kg/minute}$  or less were followed occasionally by increased clearances and a small fall in systemic pressure. The effects of adrenalin were reversed by the prior administration of Dibenamine. Arterenol at doses varying from  $\frac{1}{4}$  to 8  $\mu\text{g/kg/minute}$  uniformly reduced water and electrolyte clearances and elevated systemic blood pressure. The effects of arterenol on renal clearances were not consistently blocked by prior administration of Dibenamine in doses as high as 10 mg/kg. even in those instances in which blood pressure elevation was prevented.

*Influence of hypertonic sodium salts on renal hemodynamics and electrolyte excretion.* SAMUEL A. CORSON, ELIZABETH J. O'LEARY\* AND A. L. SIEGEL.\* Dept. of Physiology, Toledo Hospital Institute of Med. Research, Toledo, Ohio.

Hypertonic solutions of sodium salts of organic acids (0.8 M disodium succinate + 0.6 M disodium fumarate) or of chloride (2.8 M) were infused into normal unanesthetized trained or anesthetized (pentothal 20 mg/kg. or Nembutal 27 mg/kg.) dogs by means of a constant infusion pump at the rate of 1.6 ml/minute. Glomerular filtration rates (GFR) and renal plasma flow (RPF) were determined by measuring clearances of creatinine and para-aminohippurate, respectively. Sodium chloride infusion in an unanesthetized dog was accompanied by a small increase in GFR. Satisfactory RPF measurements were not made under these conditions. Disodium succinate-fumarate infusions in anesthetized and unanesthetized dogs led to a small increase in GFR, an apparent decrease in PAH clear-

ance and a consequent increase in the filtration fraction. However, preliminary renal vein catheterization experiments in anesthetized dogs indicated that succinate-fumarate infusions were accompanied by a significant decrease in the renal PAH per cent extraction, thus giving increased RPF values. Moreover, direct measurements of blood flow in the renal artery in anesthetized dogs by the thermo-stromuhr method also indicated an increase in renal blood flow accompanying succinate-fumarate infusions. The average urine sodium concentrations among the individual unanesthetized dogs following succinate-fumarate infusion varied from 150 to 280 mEq/l. Most of the sodium administered was excreted during a period of about 3 hours following the infusion. A greater proportion of the infused sodium was retained following the NaCl infusion than in the case of the organic acid salts. During the period of diuresis the dog receiving NaCl excreted considerably more chloride (448 mEq.) than sodium (258 mEq.); potassium excretion during the same period (57 mEq.) does not account for the cations required to balance the excess chloride.

*Vasodilator responses in the footpad of the dog.* J. W. COX (introduced by W. C. RANDALL) Dept. of Physiology, St. Louis Univ. Med. School, St. Louis, Mo.

In anesthetized dogs, the responses of the small vessels of the footpad to electrical excitation of the peripheral nerves and sympathetic chains have been studied by means of the photoelectric plethysmograph. Dilator responses have been recorded in 18 animals from stimulation of the tibial nerves. These responses occur despite previous section of the ventral roots from the thirteenth thoracic through the second sacral inclusive. Although dilator responses have been obtained from stimulation of the seventh lumbar and first sacral vertebral levels of the sympathetic chain, results in two animals suggest that the integrity of the lumbar sympathetic is not requisite since stimulation of the tibial nerve still elicited dilatations after verified lumbar sympathectomy. Dilatations have also been recorded in response to stimulation of the distal end of the acutely sectioned tibial nerve in footpads with all innervation sectioned. Homolateral dilator responses have never been observed by us in response to stimulation of the deep peroneal nerve; constrictor responses of submaximal magnitude are, however, readily produced by repetitive stimulation. The dilator responses observed fall into one of several types; 1) a marked and prolonged dilatation following immediately upon recovery from a brief constriction induced by repetitive shocks delivered to the tibial nerve; 2) initial dilatation which passes into a constrictor phase and which may or may not be subsequently followed by another dilator phase; and 3) most frequently, a pure dilator response to single induction shocks delivered to the intact or acutely sectioned tibial nerves. Break induction shocks

have been found to be the most reliable for elicitation of these pure dilator responses. The response latency has been found to average 1.4 seconds with a range of 0.7 to 2.5 seconds. The median of 100 determinations on 11 animals is 1.4 seconds. This differs considerably from the latency of the constrictor response, the average here being closer to 2.5 seconds.

*Comparison of Mesantoin with other anticonvulsant drugs.* PAUL F. CRANFIELD,\* HENRY M. SUCKLE\* AND O. SIDNEY ORTH. Depts. of Physiology and and Pharmacology, Univ. of Wisconsin Med. School, Madison.

Anticonvulsant and toxic doses of Mesantoin, Tridione, magnesium gluconate, and Dilantin were determined at the 66% level in Sprague-Dawley rats weighing 400 gm. or more. Drugs were administered intraperitoneally daily for 5 days. Maximal seizures were produced by electroshock (60 cycle, 150 ma., 0.2 seconds, ear electrodes). Protection was considered produced when there was abolition of fore-limb flexion in the maximal seizure, rather than abolition of hind-limb extension. A 'protective index,' toxic dose/protective dose, was calculated. Results with Dilantin duplicate the well established 50 mg/kg. protective, 100 mg/kg. toxic, P.I. = 2.0. The toxic dose of Tridione was 400 mg/kg., of magnesium gluconate 60 mg/kg. No protective, non-toxic dose was found for either of these drugs. Reports of a protective non-toxic dose of Tridione rest on single administrations, and the hind-limb extension criterion. The protective and toxic doses of Mesantoin were 50 and 150 mg/kg., respectively, the P.I. = 3.0. Protective doses of 5 mg/kg., toxic 60 mg/kg., P.I. = 12.0 have been reported, based on the hind-limb extension criterion. Our results with Mesantoin and Tridione accord with clinical experience and experiments on humans subjected to electroshock, which indicate the potency and toxicity of Mesantoin to be of the order of Dilantin, and which find Tridione ineffective against grand mal and electroshock convulsions. Drugs to be administered on a prolonged basis should not be evaluated by single administration tests alone. Abolition of fore-limb flexion may yield a criterion of protection which better predicts clinical usefulness of anticonvulsant drugs.

*Heart minute volume in 'all-out' maximal endurance exercises.* T. K. CURETON AND N. B. STRYDOM,\* R. D. MICHAEL\* AND PAUL HUNSICKER.\* Physical Fitness Research Lab., Univ. of Illinois, Urbana.

Testing a wide variety of human subjects on  $O_2$ -Intake,  $O_2$ -Debt and Grollman Minute Volume in various maximal exercises shows: 1) The oxygen A-V differences increase proportionately to the gross  $O_2$ -Intake in progressively more intensive exercises on the bicycle ergometer and on the motor driven treadmill until rates of work corresponding to 8000 ft.-lb/minute and 5 miles per hour, respectively, are performed. In

well-conditioned men performing relatively strenuous exercises the A- $VO_2$  differences become fairly constant in a relatively narrow range from 110 to 135 cc/l., with a mean at about 123 cc/l. This relationship breaks down in poorly conditioned men performing intensive exercises for the greatest length of time to apparent exhaustion. In a sample of 50 men there is no sensible rank order relationship in the obtained A- $VO_2$  differences with respect to quality of the endurance performance as measured in the length of time the exercise can be performed in 'All-Out Endurance Events.' 2) Individual differences in coordination, leg length, body build and training appear to influence the running and the A- $VO_2$  differences so that there is an insignificant correlation between these measures and the time of the 'all-out' performances on the bicycle ergometer or the treadmill. For practical testing the A- $VO_2$  differences are not absolutely needed. The variance of predictive value for fitness (duration of the exercise) is in the oxygen supply and various other elements which influence efficiency of running. 3) In 'all-out' performances of such strenuous athletic nature the  $O_2$ -Intake (l/minute)/.123 equals a close approximation of heart minute volume for well trained subjects but the blood flow is correlated only  $.50 \pm .14$  with time of the All-Out Treadmill Run, 10 mi/hr., 8.6% grade. 4) Rhythmic walking, cycling or jogging, executed as relaxed and as smoothly as possible (ballistically) without sustained tension permit the highest Arterio-Venous Oxygen Differences. Exhaustive exercises done at a high rate of speed cause a lowering of the values of the A-V Oxygen Differences. Christensen found an average of 123 cc/l. in strenuous cycling, which compares favorably with our findings in the same exercise and for champion runners on the 10 mi/hr. All-Out Treadmill Run.

*Self selection of rehabilitation diets by rats following dietary restriction.* ESTHER DACOSTA AND RUTH CLAYTON (introduced by A. J. CARLSON). U. S. Army Med. Nutrition Lab., Chicago, Ill.

One hundred eight rats were used in this study. Twelve served as controls and the remainder (3 groups of 32 each) were restricted on either a low calorie (20% protein) diet, a carrot (2% protein) diet or a synthetic vegetable (5% protein, 10% salt) diet for 13 weeks. During 13 weeks of dietary rehabilitation 24 rats from each group were offered a free choice of 3 diets, high (60%) protein, high (35%) fat and high (81%) carbohydrate. The caloric efficiency (cal/gm. gain in wt.) during the first 4 days of rehabilitation was less than when either rehabilitation diet was fed alone; however, it was greater than that of the control animals. During late rehabilitation the caloric efficiency of all experimental rats was essentially the same. During early rehabilitation the order of selection of the diets was: 1) high carbohydrate, 2) high protein and 3) high fat. As rehabilitation progressed the high-fat diet moved to first place. Although the high-fat diet remained the one

of choice, the average gains in body weight did not equal those of rats fed a high-fat diet alone. Regardless of the type of preceding dietary restriction, the average weight gains during rehabilitation were almost the same and the final weights at the end of 13 weeks were still below those of the controls.

*Cytological and respiratory effects of cyanide on tissue cultures.* BETTY DANES AND P. J. LEINFELDER. Depts. of Ophthalmology and Physiology, College of Medicine, State Univ. of Iowa, Iowa City.

This study attempts to correlate inhibition of respiration of tissue cultures by cyanide with changes in cellular activity. Heart, liver and ocular tissues of embryonic chick and newborn rat were cultured by a modified roller tube technique which permitted the tissues to be exposed to HCN. Cytologically these cultures showed that cellular migration could be unaffected, partially or completely inhibited by various concentrations. Growing chick heart cultures exposed to cyanide undergo cytolysis at concentrations that do not affect chick heart cultures exposed constantly from the time of implantation. In cultures of 7-day chick heart, 9-day chick cornea and retina exposed to HCN for the entire 24 hours of cultivation, the incidence of nuclear abnormalities such as bilobed forms increase as cellular migration is curtailed between 30 to 80%. Few nuclear abnormalities occur if cellular activity is curtailed more than 80%. Oxygen consumption of 7-day chick heart cultures was measured under control and experimental HCN conditions. Curtailment of oxygen consumption by 16% for 24 hours does not appear to affect cellular activity. Within the range of 31 to 39% depression of respiration there is an increase in cellular inhibition from 33 to 73%. Above 85% curtailment of oxygen consumption there is complete inhibition of cellular migration. After 24 hours there is no further fall in the rate of respiration.

*Influence of physiological stress upon hypertension in the rat.* H. G. DANFORD\* AND R. C. HERRIN. Dept. of Physiology, Univ. of Wisconsin, Madison.

Hypertension was produced in 125-gm. rats by wrapping silk about both kidneys. Systolic pressures were determined by a pressure cuff and tail plethysmograph. On 40 such operated rats, systolic pressures were determined weekly for 60 days. At this time, the blood pressure having stabilized, total and differential leukocyte counts were made on tail blood. Of the 40 operated rats, 32 developed a hypertension of 180 mm. Hg or more. The blood of the hypertensive rats was found to contain a significantly lower percentage of lymphocytes ( $57.3 \pm 15.9\%$ ) than was found in 43 normal rats ( $79.0 \pm 8.6\%$ ) or in the 8 rats with relatively normal blood pressures whose kidneys had been wrapped in silk ( $72.2 \pm 7.9\%$ ). No difference in the total number of leukocytes between the three groups was found; however, a significant increase in circulating eosinophils was

observed in both operated groups. A further reduction in the number of circulating lymphocytes was produced in hypertensive rats by restriction of calories, exposure to cold and deficiencies of the vitamin pyridoxine and the amino acid tryptophane. The 29 animals used were subjected to one of the above stresses for approximately 30 days. Average weight losses of 7 to 30% of the initial body weight resulted from each form of stress but no consistent change in the hypertensive blood pressure was obtained.

*Metabolism of the stomach in relation to the secretion of acid.* HORACE W. DAVENPORT. Dept. of Physiology, Univ. of Utah College of Medicine, Salt Lake City.

Mouse stomachs were incubated *in vitro* under nearly optimal conditions, and their rate of inorganic acid secretion was measured. Acid secretion was found to be a linear function of the glucose concentration of the incubation fluid up to 20 mM/l. The addition to the fluid of amorphous insulin in a concentration of one unit per ml. or higher increased the rate of acid secretion by stomachs from normal mice when the glucose concentration was 5 mM/l., but it had no effect at other glucose concentrations. Stomachs from diabetic mice secreted acid at a higher rate than those from normal mice at all glucose concentrations except zero and 20 mM/l. The addition of insulin to the fluid reduced the rates to those of normal stomachs at the same glucose concentrations. Insulin reduced the rate of secretion by normal stomachs below that of the controls when the substrate was 10 mM lactate. With the same substrate stomachs from diabetic mice secreted more acid than stomachs from normal mice, and the addition of insulin reduced the rate of secretion. Stomachs from normal and diabetic mice with and without insulin secreted acid at the same rate in the presence of 10 mM pyruvate or acetoacetate. The latter substance is almost as effective a substrate for acid secretion as is glucose.

*Refractory period and equilibration in nerve.* M. M. DAVIS AND R. W. GERARD. Univ. of Chicago, Chicago, Ill.

The refractory period of frog sciatic has been studied in terms of the time curves of return, after a full response, of spike height, excitability for threshold and maximal responses, and conduction velocity. The prolongation of these recovery curves after two or more responses, within the relative refractory period, has also been followed, as an index of equilibration. Spike height is 10 to 20% normal at the end of the absolute refractory period (ARP = 1.2 msec.) and recovers over a steep logarithmic curve to 90% normal at 3.5 msec. (22°C.). Excitability, both for threshold and maximal response, is 40% normal at the end of the ARP and recovers over a more gradual curve to 90% normal at 6 msec. Conduction velocity is, surprisingly, 70% normal at the end of the ARP and recovers linearly to 90% normal at 7 msec. Following two responses at a 2-msec. interval,

the recovery curves (mainly of responsiveness) are prolonged 10 to 20% less at shorter intervals; and additional responses (up to 10 shocks at 750/sec.) lead to further slowing of recovery, each response adding a smaller increment of delay than preceding ones, up to a 20 to 40% prolongation. Equilibration thus sets in at once, as expected from the metabolic interpretation of it.

*Electrical registration of knee jerk and ballistocardiogram.* (Demonstration). ADAM B. DENISON, ALFRED W. RICHARDSON\* AND HAROLD D. GREEN. Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

The knee jerk is recorded by a sensitive pneumograph strapped about the thigh and connected, by an air transmission system, with an arterial type (P23A) Statham gage. The movements of the ballistocardiographic table are transmitted directly to a sylphon bellows, which is connected by a fluid system to a venous type (P23B) Statham gage. In either case the gage is connected to a Brush strain analyser and Brush ink writing magnetic penmotor recorder. These systems are convenient, flexible and give results fully as satisfactory as photographically recording systems.

*Implications of cholesterol-ascorbic acid antagonism.*

FRANCES M. DENT, WALTER M. BOOKER, RAYMOND L. HAYES, WARREN HARRIS AND SOLOMON GREENE. Dept. of Pharmacology and Oral Medicine, Howard Univ. Colleges of Medicine and Dentistry, Washington, D. C.

The following experiments were designed: 1) Daily administration of cholesterol to dogs, studying the effects on the plasma and blood cell ascorbic acid, and the effect on the 24-hour excretion of ascorbic acid in dogs and in rats. 2) The acute effects of cholesterol (4 hours) on the plasma and blood cells in dogs. 3) The effects of the daily administration of cholesterol alone and with vitamin C on the storage of vitamin C at the adrenal gland, brain, liver, kidney, muscle, fat and tendon in rats. 4) The modification of the cholesterol-vitamin C relationship by DCA, ACE and Cortisone regarding daily urinary excretion of ascorbic acid, fecal excretion of cholesterol and tissue distribution of cholesterol and of vitamin C in rats. Our results indicate that: 1) cholesterol depresses remarkably the plasma and blood cell levels of ascorbic acid in both day-to-day and acute experiments. 2) Animals administered cholesterol daily show an increased excretion of ascorbic acid in the urine. 3) Although ACE and cortisone are more effective in increasing the storage of vitamin C in certain tissues, particularly the adrenal gland DCA is more effective in blocking the depressing effect of cholesterol on the plasma and blood cell ascorbic acid, and on the influence of cholesterol on the urinary output of ascorbic acid. The apparent cholesterol-ascorbic acid antagonism is not complete through-

out all tissues studied. In brain particularly, both cholesterol and vitamin C are elevated over their control counterparts. This, of course, may be compensatory.

*Release of adenine derivatives from mammalian blood cells following admixture of blood with hypertonic solutions.* INGRITH J. DEYRUP. Depts. of Zoology of Barnard College and Columbia Univ., New York City.

When a strongly hypertonic solution mixed with homologous blood is injected i.v. into anesthetized dogs or rabbits, circulatory disturbances occur and are far more severe than the effects of administration of corresponding amounts of the hypertonic solution alone. This has been attributed to the release of a pharmacologically active substance from blood cells exposed to hypertonic solutions (*Am. J. Physiol.* 160: 509, 1950). In an attempt to identify this substance, ultraviolet absorption measurements have been made, by means of a Beckman universal spectrophotometer, on fluids derived from mixtures of guinea pig, rat or human blood with 50% glucose or 10 to 20% NaCl. Such fluids, after precipitation of the blood proteins with 1.5 to 6% perchloric acid were found to have a characteristic ultraviolet absorption spectrum identified by its shape and peak at about 260 mμ as the absorption curve of adenosine and its phosphate derivatives. Estimates of the amounts of adenine compounds released from the blood cells in the conditions of the experiment were made. It was concluded that these compounds were present in sufficiently high concentrations to account quantitatively for the effects of fluids derived from mixtures of blood and hypertonic solutions on the circulation of mammals, and on the guinea pig intestine *in vitro*.

*Energy expenditure in bicycle riding.* D. B. DILL, J. C. SEED AND F. N. MARZULLI. Army Chemical Center, Md.

Despite the physiologist's common use of the bicycle ergometer, he has paid little attention to the physiology of bicycle riding. This is partly because the L. Zuntz monograph on energy exchange in bicycle riding, published 50 years ago, covers the subject so well. Practical experience of two of the authors (JCS and DBD) engendered an interest in the effect of a modern innovation, increased cross-section of tires, on energy expenditure. Paired outdoor experiments were undertaken with the two subjects keeping pace beside an automobile. Expired air was led to Douglas bags in the automobile. Wind and ground speeds were recorded. One bicycle had tires 26 x 2½ inches, the other 26 x 1½ inches; they were used alternately by the two subjects. With ground speed varying from 150 to 225 m/minute and with winds of from -77 to +225 m/minute the net oxygen consumption is about 190 cc/minute greater for the rider on the large-tired bicycle. In easy riding about one-half more energy is expended by the rider on the large-

tired bicycle. The expenditure is about one-fifth greater for large tires in moderate riding and about one-tenth greater in hard riding. Many other considerations than tire cross-section enter into the choice of a bicycle. However, these results indicate that the large tires commonly provided on American bicycles and rarely on European bicycles, are undesirable for the adult who uses a bicycle to get from one place to another with minimal energy expenditure.

*Influence of stimulus pattern on reflex deglutition.* R. W. DOTY (introduced by R. W. GERARD). Dept. of Physiology, Univ. of Chicago, Chicago, Ill.

De-glutition evoked in the dog by superior laryngeal nerve stimulation is relatively indefatigable, 1500 swallows in an hour of continuous stimulation. An optimal stimulus frequency, at which deglutition is elicited with the shortest latency, lowest threshold, and most rapid continuing rate, is species characteristic (dog, 20-30/sec.; rabbit 60-80; cat 30-50). A limiting frequency (dog 50-100), above which the reflex is unobtainable with moderate stimuli, is precise to within 3/second for any given condition, but varies, as does the latent period (0.5-60 sec.), but not the optimal frequency, with the time since preceding swallow, intensity of stimulation, rapidity of respiration, and depth of anesthesia, and under strychnine or myanesin. The latent period for a second ipsilateral excitation is roughly proportional to time (up to 3 min.) since cessation of the first, whereas contralateral facilitation disappears in about 10 seconds. Concurrent stimulation of one superior laryngeal above limiting frequency does not affect the response to optimal stimulation of the other (or of a glossopharyngeal) but central summation results on stimulation of both superior laryngeals at subliminal frequency or intensity. Block with rapid stimulation is this upstream to the crossed summation. The optimal response is remarkably independent of temporal patterning of the stimuli, e.g. shocks at intervals of 10 msec.-90 msec.-10 msec. . . (continued) 50-50-50-50- . . . (continued), or 33-33-33- . . . (on for 1452 msec., off for 1548), all gave like responses. The CNS seems to count and add pulses within spans of hundreds of milliseconds.

*Effect of epinephrine and insulin on plasma potassium in rats.* ABRAHAM DURY. Dorn Lab. for Med. Research, Bradford Hospital, Bradford, Pa.

Although the level of plasma potassium has been reported to be decreased after the injection of epinephrine or insulin, desoxycorticosterone is considered the humoral agent regulating normal levels of blood sodium or potassium. Insulin and epinephrine, particularly, have been shown to influence adrenocortical secretion. The present study was undertaken to determine whether insulin and epinephrine affect the level of blood potassium via modification of adrenocortical activity or was a physiological property of these two

substances. Groups of normal intact, and adrenal demedullated and adrenalectomized rats two weeks post operative were injected as follows: Physiological saline (0.5 ml., s.c.); epinephrine (0.02 mg/100 gm. of rat, s.c.); insulin, regular (0.5 U, s.c.); and insulin (0.25 U, s.c.) followed 5 minutes later by epinephrine (0.02 mg/100 gm. of rat, s.c.). Determinations of the plasma potassium level and glucose were made on blood obtained by cardiac puncture 60 and 180 minutes after the injections. Statistically, significant depression of the plasma potassium level was found in the demedullated and adrenalectomized groups 60 minutes after epinephrine, and after insulin-epinephrine. The plasma potassium level was not changed in any of the groups after insulin alone. In the groups of rats 180 minutes after the injections a statistically significant depression in the plasma potassium level was induced after epinephrine in the demedullated group and after insulin in the intact group. These results show that epinephrine can depress the level of plasma potassium without the mediation of adrenocortical activity; and that insulin apparently influences the level of plasma potassium via 'reflex' epinephrine activity.

*Changes in antidiuretic activity of rat serum after X-irradiation.* ABRAHAM EDELMANN AND W. J. EVERSOLE. Brookhaven Natl. Lab., Upton, L. I., N. Y. and Syracuse Univ., Syracuse, N. Y.

The experiments reported here concern an attempt to determine the physiology involved in the previously reported (*Federation Proc.* 8: 39, 1949) diuresis occurring within 24 hours after whole body x-irradiation of the rat. A test dose of water, 4 ml/100 cm.<sup>2</sup> body surface was given by stomach tube 30 minutes or 24 hours after whole body x-irradiation (160 KVP 10 ma. 0.2 mm.<sup>2</sup>). Control animals were similarly treated except they were not irradiated. Exposed animals excreted the administered water at a more rapid rate than the controls. Serum taken from irradiated rats at various times after exposure was tested for antidiuretic activity by the method of Birnie *et al.* (*Proc. Soc. Exper. Biol. & Med.* 70: 83, 1949). Significant decreases in antidiuretic activity were found at 12 and 24 hours after irradiation. These results indicate that a decrease in amount of circulating antidiuretic substance or substances is a factor involved in the increased urine flow after irradiation. (Research carried out at Brookhaven Natl. Lab. under the auspices of the U. S. Atomic Energy Commission.)

*Further studies on the regional distribution of heat losses.* H. E. EDERSTROM, A. B. HERTZMAN AND W. C. RANDALL. Dept. of Physiology, St. Louis Univ. School of Medicine, St. Louis, Mo.

Young male subjects were exposed nude to environmental temperatures ranging from 23° to 38°C. for a 3-hour period. Recordings of skin temperatures of the forehead, cheek, chest, abdomen, upper and lower arm, thigh, calf, sole and palm were made with thermo-

couples. Sweat output was found by the weighed desiccating capsule technique. Calculations of evaporative, radiative and convective heat losses were made in the usual way from the thermal data. At low environmental temperatures radiative and convective heat losses showed considerable spread over the local surface areas, with the highest losses appearing in the head and trunk regions, and the lowest in the palm and sole. At the higher temperatures heat losses were approximately the same in all areas. Evaporative heat losses were fairly uniform over all areas at low temperatures, but in the high ranges the leg, trunk and forehead were the predominant avenues of heat loss. The total effective heat losses from the surface areas listed above were weighed according to the fraction of total body surface that each represented. The results disclosed that the trunk and leg were the major areas of heat loss in every temperature range investigated. The head and arm were found to be areas of relatively low heat loss, while the palm and sole were consistently the areas of lowest heat loss in all temperature ranges. Calculation of the total effective heat loss of the entire body surface was done from the local area data. Values within the expected range were found, indicating the accuracy of the method of partitioned calorimetry.

*Abnormality of regulation of heart rate and peripheral resistance after damage to the aortic valves.* R. G. ELLISON AND R. W. PICKERING (introduced by W. F. HAMILTON). Depts. of Thoracic Surgery and Physiology, Med. College of Georgia, Augusta.

Aortic regurgitation was established by tearing the valves with a sound pushed down the carotid artery. In confirmation of earlier work (*Am. J. Physiol.* 107: 436), it was found that raising the blood pressure by epinephrine to figures such as 300/150 no longer gave the usual cardiac slowing. Lowering the blood pressure with acetylcholine etc. quickened the heart in less than normal proportion. Raising the blood pressure did not give the usual vasodilation in the auto-perfused leg guarded against direct action of the drug by interposing a long tube in the perfusion stream. Failure of reflex cardiac slowing is due to functional failure of the afferents because stimulation of the vagus causes the usual inhibition. The failure results immediately after the establishment of the regurgitation and ceases as the lesion heals. The reflex slowing is also lost when an artificial patent ductus or abdominal A-V fistula is established. On the other hand the heart rate is related to mean pressure in the perfused carotid sinus regardless of whether the perfusion is under steady or pulsatile pressure. A clinical case of aortic regurgitation failed to show cardiac slowing in response to the rise in pressure induced by neosynephrine or by the Valsalva experiment. A clinical case of patent ductus showed slowing in response to these same maneuvers which was unchanged after division of the ductus arteriosus.

*Response of non-innervated smooth muscle to tension.*

JOHN FERGUSON. Dept. of Physiology and Pharmacology, Creighton Univ. School of Medicine, Omaha, Nebr.

A study was made of the effect of tension on motility of non-innervated smooth muscle. The amnion of a developing chick (11-17 days) was suspended as a muscle strip in warmed aerated Sollmann-Rademaekers' solution and the motility recorded on a smoked drum. This structure contains muscle but is thought to be devoid of nervous elements of any description. Different degrees of tension, ranging from 20 mg. to over 1 gm., were applied to be quiescent and to the spontaneously rhythmic muscle. After the preparation had been adjusted in the solution from 30 to 60 minutes a response was obtained in the majority of cases when the muscle was stretched and released. A response was obtained also with change of tension,—either increased or decreased. Over 100 determinations to changes in tension were made on amnions of 11 to 17 days. To increased or decreased tension the quiescent muscle responded by one or more contractions, and the rhythmic muscle responded by an immediately augmented amplitude of contraction. Ethisterone (anhydrohydroxyprogesterone) or increase of potassium ions in the solution appeared to be conducive to, and deficient aeration unfavorable to, obtaining a response to stretch or release. For rhythmic activity there is apparently an optimum tension for each amnion,—this varies from 50 to 100 mg.

*Electrical studies of injury produced in guinea pig's ear by strong high-frequency sounds.* C. FERNÁNDEZ,\* H. VON GIERKE,\* D. H. ELDRIDGE, JR.\* AND H. DAVIS. Aero-Med. Lab., Air Materiel Command, Wright Patterson Air Force Base, Dayton, Ohio, and Central Institute for the Deaf, St. Louis, Mo.

Intra-cochlear electrodes were implanted in scala tympani and scala vestibuli of turn 1 and in scala vestibuli of turn 4 (guinea pigs). Test stimuli were tone-pips of 8000, of 2000 and of 500 cps (20 pips per second) and 900 cps pure tone. (Tone-pips do not equilibrate or fatigue the action potentials.) By subtracting the ST response from SV the action potentials were cancelled. By adding the responses the cochlear microphonics were cancelled. Thresholds for each type of response were thus determined separately. Loud tones at 9000 cps were produced by a rod electro-magnetically driven at its resonant frequency. During exposure (7-480 sec.) a strong subharmonic (4500) cps could be heard directly and also appeared in the cochlear microphonic. At 126 db and higher the subharmonic nearly always was modulated or became intermittent and 'crackling.' A clear division appeared between mild impairment (a mere elevation of thresholds with stabilization and sometimes slight recovery) and severe injury (complete although sometimes delayed disappearance of action potentials at 8000 cps, great loss at 2000 cps, and great reduction in cochlear microphonics, particularly at

8000 cps). Whenever the product of sound pressure (in microbars) by duration (in seconds) exceeded a particular value (about 30,000) the injury was severe. Severe injury was caused by 120, 126, 130 and 140 db re 0.0002 microbar for 480, 120, 60 and 20 seconds, respectively. (Part of this work supported by Office of Naval Research under Contract N6onr-272 with Central Institute for the Deaf.)

*Microscopic observation of locally burned blood and vessel walls.* ROY P. FINNEY, JR. (introduced by M. H. KNISELY). Dept. of Anatomy, Med. College of the State of South Carolina, Charleston.

The purpose of the investigations here reported was to determine the visible responses of small blood vessels and flowing blood to the minimal thermal burns to which these would respond. Transilluminated mesenteric vessels were heated by radiant heat from a microcautery consisting of a 36-gauge platinum wire loop electrically heated. The wire never touched the tissue, thus eliminating possible reactions from mechanical stimulation. Cats, dogs, guinea pigs, rats and mice have been used. Microscopes focused on the small vessels permitted continuous observation of the area before, during and after the application of heat. Five separate reactions have been observed: 1) contraction of vessels; 2) dilation; 3) formation of white thrombi and emboli, composed of as yet unidentified substances; 4) sticking of a thin layer of red cells to the inside of the vessel wall, and 5) formation of thick sludge in the blood passing the burned areas. Within a few seconds after a micro-burn is made, white thrombi begin to form on the inside of the vessel wall. A thrombus may be a thin white layer, or it may form masses so large that the flow of blood is almost completely stopped. (Histological sections indicate that these thrombi are composed of a granular substance which looks like aggregates of platelets.) Emboli are formed when chunks are broken from fixed thrombi and swept away in the blood stream. Building up and tearing down of thrombi may continue from a few minutes to several hours. With minimal burn the formation of thrombi stops and all of the material is swept away leaving a normally appearing vessel.

*Metabolic effects of an insulin-free pancreatic extract.*

PIERO P. FOÀ AND HARRIET R. WEINSTEIN.\* Dept. of Physiology and Pharmacology, Chicago Med. School, Chicago, Ill.

Ten alloxan diabetic dogs have been kept in good condition without insulin for months. They excreted from 25 to 100 gm. of glucose per day, but developed only a mild ketosis. Possibly glycosuria of alloxan diabetes is great not only because insulin is lacking, as it is in pancreatic diabetes, but also because the undamaged  $\alpha$ -cells secrete a hyperglycemic factor. No explanation however exists for the lack of severe ketosis. Ketosis was produced in dogs by fasting, alloxan,

or pancreatectomy. Pancreatic glands from alloxan diabetic dogs were extracted as done for the preparation of insulin and the '15% cake' dissolved in saline and injected intravenously into 9 dogs (10 gm. of pancreas/kg.). The blood glucose rose about 30% in 15 minutes returning to normal in about 1 hour. The blood ketones decreased about 30% in 15 minutes and remained low for at least 3 hours. Similar results were obtained with a 15% cake from normal pancreas (E. Lilly & Co.; 6 dogs), after inactivation of insulin, and with a similar extract of gastric mucosa of alloxan diabetic dogs (4 experiments). Although a lowering of the ketonemia was obtained also with glucose (1 gm/kg. i.v.; 2 dogs), hyperglycemia may not have been the only cause of the hypoketonemia following pancreatic extracts, as a purified hyperglycemic factor (E. Lilly & Co., 1 mg/kg.; 2 dogs) produced a marked hyperglycemia (up to 70%) and a rise not a drop in ketonemia (25%). It is suggested that the extracts may contain a substance capable of lowering the blood ketones.

*Measurement of uneven alveolar ventilation.* W. S. FOWLER, E. R. CORNISH, JR.\* AND S. S. KETY. Dept. of Physiology and Pharmacology, Graduate School of Medicine, Univ. of Pennsylvania, Philadelphia.

During the elimination of pulmonary  $N_2$  occurring in the first several minutes of oxygen inhalation, measurements were made of tidal volume, number of breaths and mean  $N_2$  concentration of each expiration, the latter from continuous and simultaneous analysis of expiratory volume flow and  $N_2$  concentration (Lilly-Hervey  $N_2$  meter) of expired gas. These measurements indicate the presence of uneven alveolar ventilation in both healthy persons and patients with chronic pulmonary disease. Equations were developed for a one, two, or three-phase system having one total tidal volume, the mean expired  $N_2$  concentration of which is a function of all the ventilating phases. Solution of the equation gives values for lung volume (functional residual capacity) and effective tidal volume (tidal minus dead space) for each phase. An equation for at least two phases is required to describe the records of most persons. The extent of non-uniformity of alveolar ventilation can be quantitatively expressed as deviations (increase in the average number of breaths a  $N_2$  molecule remains in lungs) from that value which would obtain if alveolar ventilation were uniform. Patients with chronic pulmonary disease have less uniform alveolar ventilation than healthy persons.

*Effect of beta radiation on gastric secretion in dogs.* B. W.

FOX, A. LITTMAN, J. LASH AND M. I. GROSSMAN. Dept. of Clinical Science, Univ. of Illinois, Chicago.

Radium, Ruthenium 106-Rhodium 106 and Strontium-90 have been used for intragastric radiation in dogs. The radioactive source in each case was mounted as an intact essentially point source, introduced within the stomach for a period of exposure and then with-



drawn. Effects on acid secretion were evaluated by serial histamine gastric analyses. Radium was used as a 50-mg. source. The distance to the gastric mucosa in this as well as other work here reported was estimated to be 5 cm. Four dosage levels ranged from 1500 to 9000 mg. hours. This included the lethal dose. Suppression of acid secretion was obtained with increasing doses. The lethal dose was reached without producing achlorhydria. Ruthenium 106-Rhodium 106 was used as a 125-millicurie source. Four dosage levels ranged from 1½ to 24 hours. The dose was approximately 1000 to 1500 rep per hour. Six hours and over produced death. Achlorhydria was achieved only with lethal doses. Whether a level under the lethal dose can produce achlorhydria is being investigated. Strontium 90 was used as a 125-millicurie source. Six dosage levels ranged from 60 to 96 hours. The dose was approximately 200 to 250 rep per hour. Twenty-four hours and over produced temporary achlorhydria; 96 hours may be lethal. This preliminary work compares gamma and beta sources, and establishes general response patterns to increasing doses. Both gamma and beta rays produce suppression of acid secretion, beta producing more marked effects within tolerance doses. Work is being continued to define better the margins of safety, and to try to establish a dose or schedule of cumulative doses capable of producing permanent achlorhydria.

*Effect of adrenalectomy and DCA on radioisotope intramuscular clearance and distribution in the rat.* FREDERICK R. FRANKE, JOSEPH B. BOATMAN, THOMAS LONGABAUGH, T. R. KENDRICK AND R. S. GEORGE. Addison H. Gibson Lab. of School of Medicine of Univ. of Pittsburgh, Pittsburgh, Pa.

The effects of adrenalectomy and the administration of DCA and saline on the clearance of intramuscularly injected  $I^{131}$  and  $Na^{24}$  in the rat were studied as well as the distribution of  $I^{131}$  in various organs in these altered states. Four groups of 15 albino rats were used; *group I*, sham operated, *group II* adrenalectomized, *group III* adrenalectomized and given 0.5 mg. DCA in oil daily, and *group IV* adrenalectomized and given DCA similarly plus 2% saline as drinking water. Interval determinations of the rate of local clearance of  $I^{131}$  and  $Na^{24}$  were made using a Geiger-Muller tube coupled to an auto scaler. The decline in activity was expressed as the percentage of activity remaining at the site per unit time considering the activity in counts per minute at initial time as 100%. Approximately 3 to 5 microcuries of  $Na^{24}$  and 10 to 25 microcuries of  $I^{131}$  in physiological saline in 0.05 ml. were used for injection into the hamstring muscles. At sacrifice 200 microcuries of  $I^{131}$  was injected intraperitoneally and its distribution in pituitary, thyroid, liver and muscle was determined by ashing technic. At the end of a 15-minute observation period the intramuscular  $Na^{24}$  clearance rate was 55.4% activity remaining for *group I*, 63.5% remaining for *group II*, 42.4% remaining for *group III* and 67.7%

activity remaining for *group IV*. For a similar period the  $I^{131}$  clearance rate was 24.4% activity remaining for *group I*, 30.1% for *group II*, 20.5% for *group III* and 22.1% activity remaining for *group IV*. There was a progressively decreased weight of both pituitary and thyroid glands in the experimental groups compared to those of the sham series. In *group IV* both pituitary and thyroid tissue had increased concentrations of  $I^{131}$  over the concentrations in the sham-operated *group I*. (This study was aided in part by a grant from the Sarah Mellon Scaife Foundation.)

*Three-dimensional vector ballistocardiography.* SANFORD A. FRANZBLAU, WILLIAM R. BEST, JOHN P. MARGARER AND VICTOR GUILLEMIN, JR. Dept. of Medicine and Aeromedical and Physical Environment Lab., Univ. of Illinois College of Medicine, Chicago.

The conventional ballistocardiogram taken in the cephalocaudal axis might be augmented by a study of motions in the side-to-side and dorso-ventral axes, just as information obtained from a single lead of an electrocardiogram may be augmented by study of other leads. Since there is, as yet, no device for recording this behavior in three axes simultaneously, we have undertaken to record ballistocardiograph motion in the three axes consecutively, using a Nickerson ballistocardiograph. This was accomplished by the construction of an auxiliary turntable top for the conventional ballistocardiograph so that the component displacements in three dimensions could be aligned with the axis of movement of the conventional table. The three vector components were then united on a set of rectangular coordinates and the results at successive points of the cardiac cycle plotted. A wire model of this tri-dimensional vector ballistocardiogram with time markings was constructed to elucidate the pattern of motion. Inverse models of the table displacement, giving a pattern related to changes in force of the moving mass have also been prepared. A statistical analysis of 18 three-dimensional ballistocardiograms, on 12 normal subjects with standard deviations at each .04 seconds of the cardiac cycle showed that, 1) the tracings were reproducible within close limits, 2) the I-J stroke in the frontal plane was generally directed from right footward to left headward. The J-K stroke was generally directed from left headward to right footward.

*Gastric secretion in the cat.* M. H. F. FRIEDMAN, Jefferson Med. College, Philadelphia, Pa.

Experiments were performed on unanesthetized cats equipped with permanent gastric fistula and on cats under various anesthetics. In the fasting animal there was an apparently continuous gastric secretion at a slow rate which consisted of a slightly viscid, alkaline (pH > 7.2) fluid often having a chloride concentration of about 170 mEq/l. and moderate peptic activity. Acid secretion occurred only in response to specific stimuli. 1) Electrical excitation of the vagus in cats

under chloralose-urethane produced a secretion of high peptic activity but the acid concentration was never found to exceed 150 mEq/l. This secretion could be inhibited by atrophine. 2) The augmented secretion following histamine administration at first consisted of alkaline fluid which was only gradually replaced by acid. Even with the most rapid rates of secretion the acid concentration never exceeded 155 mEq/l. The chloride, however, of both alkaline and acid secretions was usually maintained at a relatively constant concentration of about 170 mEq/l. The peptic activity of the juice was consistently low. Atropine and antihistaminic drugs did not affect histamine-induced secretion. 3) In unanesthetized cats insulin-induced hypoglycemia provoked a profuse secretion of acid juice of high peptic activity which could be abolished by atropine. This finding does not support the view that sympathetic rather than parasympathetic excitation is predominant in the unanesthetized cat under the influence of insulin hypoglycemia. In cats under sodium pentobarbital (Nembital) anesthesia, however, insulin was without effect on the secretion even though the blood sugar levels were depressed to 35 mg. %.

*Action of adrenal cortical steroids and nor-epinephrine on vascular responses of adrenalectomized rats.* I. FRITZ AND R. LEVINE. Dept. of Metabolic and Endocrine Research, Med. Research Institute, Michael Reese Hospital, and Dept. of Physiology, Univ. of Chicago, Chicago, Ill.

Recent work from this laboratory (See Ramey *et al.*, *Am. J. Physiol.* 162: 10, 1950) has shown that the well known fatigability exhibited by adrenalectomized animals is due to a circulation which becomes inadequate during performance of work. Fatigue is preceded by a fall in blood pressure and work performance is restored by temporarily normalizing the pressure level. We have found more recently that the adrenalectomized animal becomes relatively insensitive to nor-epinephrine (unpublished data). In order to evaluate the vascular reactions of adrenalectomized animals more intimately we have utilized the Chambers-Zweifach rat meso-appendix preparation. The data demonstrate that: 1) The vasoconstriction response to topical nor-epinephrine becomes impaired during stress in the adrenalectomized rat. In addition, repeated nor-epinephrine application is followed by evidence of damage to the vessels. Sensitivity is completely restored by the local application of ACE. 2) Subcutaneous injection of formalin produces complete stasis in the adrenalectomized and no visible vascular effects in the normal rat. 3) The first stage of Dibenamine block can be overcome by parenteral ACE in both normal and adrenalectomized animals. This work is being continued on the hypothesis that cortical steroids are necessary for the response of small vessels to physiological vasoconstriction.

*Physiological evidence for cochlea-cochlear pathway.* ROB-

ERT GALAMBOS, WALTER A. ROSENBLITH\* AND MARK R. ROSENZWEIG.\* Psycho-Acoustic Lab., Harvard Univ., Cambridge, Mass.

When an electrode is placed on the round window of the cochlea of a cat, clicks to either ear produce recordable electrical potentials. Clicks to the ear ipsilateral to the recording electrode arouse the familiar complex of microphonic and nerve potentials. Contralateral clicks evoke potentials somewhat later in time and smaller in magnitude. The potential evoked by contralateral clicks disappears permanently when the contralateral cochlea is destroyed. It can be reversibly reduced by temporary cooling of the contralateral cochlea, an operation that also reduces discharges in the contralateral eighth nerve. A prior click delivered to the contralateral ear depresses the neural events expected from an ipsilateral click; the time relations involved suggest a minimum conduction time of one msec. for the pathway concerned.

*Reversibility of mercurial inhibition of invertase.* CHALMERS L. GEMMILL AND E. M. BOWMAN.\* Dept. of Pharmacology, Univ. of Virginia Med. School, Charlottesville.

Previous experiments (*Federation Proc.* 9: 274, 1950) have demonstrated the extreme sensitivity of mercurials of pharmacological interest on invertase activity. Analysis of the relationship of the inhibition to concentration of sucrose indicated competitive inhibition. Numerous experiments were made with various compounds to reactivate the mercuric chloride inhibition of invertase. 2,3-Dimercapto-propanol reactivated the mercuric inhibition when the molar concentration was approximately 10 times that of mercuric chloride. Guanidine had a slight reactivator effect. When cysteine was used an augmentation of the mercurial inhibition was obtained. Glutathione acted like cysteine by increasing the inhibition. Urea, methionine and sodium thioglycolate had no effect on this reaction.

*Preparation and activation of plasma proenzyme.* EARL B. GERHEIM AND ANDRE M. WEITZENHOFER.\* Division of Basic Sciences, Univ. of Detroit, and Wayne Univ. College of Medicine, Detroit, Mich.

A purified and highly reactive proenzyme has been prepared from human plasma. The procedure: 1) dialysis against demineralized water to obtain the euglobulin fraction, 2) euglobulin treated with multiple lots of magnesium hydroxide paste, 3) non-adsorbed portion fractionated with saturated ammonium sulfate, retained 28-34 fraction for isolation of proenzyme, 4) 28-34 fraction given multiple treatments with magnesium hydroxide paste, 5) non-adsorbed portion dialyzed and dried by ether-acetone technic. Assay as to potency carried out by fibrinolytic test set up to consist of 0.2 ml. 1% fibrinogen, 0.2 ml. activator, 0.2 ml. 25 u/ml. thrombin and 0.2 ml. serially diluted proenzyme, starting with one %. Tubes read for complete lysis at 24 hours.

Potency expressed in dry weight of proenzyme lysing 0.25% fibrinogen. Average value for this has been found to be  $4.88 \times 10^{-4}$  mg. with a high of  $9.53 \times 10^{-7}$  mg. with the best activator, Lederle streptokinase. Activators in order of increasing efficiency: staphylokinase, streptokinase prepared by modification of Tillett and Garner, and Lederle streptokinase.

*Uptake of maternally administered radioactive iodine in the thyroids of newborn rats.* JOHN GERSTEN, RALPH A. KNOUFF AND GEORGE M. CURTIS, Dept. of Anatomy, Ohio State Univ., Columbus.

Radioiodine ( $I^{131}$ ) was given by injection to pregnant rats. The newborn were sacrificed before nursing. Thyroid radioautographs revealed a substantial collection of radioiodine in their thyroids. Radioiodine was also given by injection to lactating rats. The newborn were sacrificed after nursing a variable number of times. Their thyroids also showed a substantial uptake of the tracer radioiodine. Visual evidence is presented of uptake of the maternally administered radioiodine in the thyroids of the pre-nursing and post-nursing groups of newborn rats by means of radioautographs.

*Uptake of  $P^{32}$  by skeletal muscle under various experimental conditions.* D. L. GILBERT,\* C. D. JANNEY\* AND H. M. HINES. Dept. of Physiology, State Univ. of Iowa, Iowa City.

A study was made of the amount of  $P^{32}$  uptake by gastrocnemius muscles of adult rats under various experimental conditions in which the muscle of one limb served as the experimental and that of the contralateral limb as its control. The muscles were removed for study 30 seconds after an intraperitoneal injection of 0.4 millicuries of  $P^{32}$ . Under these conditions the principle variables were the volume of blood flow and the area and permeability of the capillary bed. The  $P^{32}$  uptake was the same in the right and left gastrocnemii of normal control animals. Vigorous tetanic contraction of muscle was accompanied by a decreased uptake of  $P^{32}$ ; whereas during the period of relaxation and recovery the uptake was greater than in the controls. Immobilized muscle took up less  $P^{32}$  than did their controls. The total phosphate uptake was unaffected by tenotomy but the uptake on a gram-weight basis was increased. Denervation was followed by a decreased uptake of  $P^{32}$ , the amounts of which roughly paralleled the degree of atrophy. However, in the longer periods of atrophy the uptake per gram was found to be increased. The condition of prolonged shortening or spasticity in muscle was accompanied by a decreased uptake of  $P^{32}$ . This effect was abolished by deep anesthesia and lessened by daily treatments with stretching. It is postulated that a reduced effective circulatory transfer may contribute to the atrophy and functional impairment found in spastic muscle.

*Reaction of muscle to quick stretch.* A. S. GILSON, JR.

Dept. of Physiology, Washington Univ. School of Medicine, Saint Louis, Mo.

If the length of a resting muscle already above just-stretched length is quickly increased by a small amount there is a prompt increase of tension followed by a certain loss of tension from the peak value. If the muscle is stimulated and allowed to develop some twitch tension before stretch is applied, the resulting quick increase of tension equals that for resting muscle at the same pre-stretch tension. If the stimulated muscle has been stretched during the contraction phase to tension less than tetanus tension the isometric twitch will continue to develop along a course which is that for a muscle stimulated at correspondingly high initial tension. During relaxation, tension will fall to a final level which is that found when the same stretch is applied to resting muscle at the same initial tension. If the muscle is initially at less than just-stretched length the results may be more complicated quantitatively but will show essentially the same phenomena. The tension increase as reaction to quick stretch depends upon existing tension, be the muscle resting or active. The further tension course of the twitch contraction already initiated depends upon the tension resulting from the stretch. There is no indication of increased extensibility of the muscle during activity nor of a sudden change of mechanical state at the end of the latent period. The reaction to quick stretch is not described by the usual tension-length diagram for a muscle responding isometrically.

*Further observations on action of insulin on transfer of hexoses across cell membranes: relation to adrenal cortex.* M. S. GOLDSTEIN, B. MENDEL AND R. LEVINE. Dept. of Metabolic and Endocrine Research, Med. Research Institute, Michael Reese Hospital, Chicago, Ill.

We have recently demonstrated that in eviscerated-nephrectomized animals galactose is not metabolized. A single dose (i.v.) establishes a constant blood level when its distribution is complete and an equilibrium reached. Insulin, under these conditions, causes a wider distribution of the galactose leading to a lower constant blood level. On the basis of this phenomenon we have proposed that the primary action of insulin is to facilitate the entry of unchanged hexoses into certain cells, the subsequent fate of the hexoses depending on the presence and state of activity of intracellular enzymes. Since the  $C_{11}$  oxysteroids are well established as antagonists of insulin action in the intact animal, we have investigated the possible effects of cortisone and of adrenalectomy on this action of insulin. Normal dogs were given cortisone (10-25 mg/day) for 3 days. On the second day, they were resistant or unresponsive to insulin (1/10 u/kg/i.v.). On the third day of cortisone treatment these animals were subjected to evisceration and nephrectomy. The distribution of galactose (1 gm/kg.) was measured both with and without added insulin

(1  $\text{U/kg/hr.}$ ). The administration of cortisone did not exert any measurable effect either in the presence or absence of added insulin. The distribution curves obtained could be superimposed upon those of our previous normal untreated series. An adrenalectomized dog, maintained in electrolyte balance with DCA, was eviscerated and nephrectomized and the distribution of galactose was found to be the same as in normal animals without added insulin. Thus, despite the insulin resistance of the intact cortisone-treated dogs and the increased insulin sensitivity of the intact adrenalectomized dog, the peripheral action of insulin which we were studying was not affected. These results are consistent with the view that the antagonism of the adrenal cortex to the action of insulin is not located in the extrahepatic tissues.

*Reversals of cochlear response patterns with death of the animal.* ROBERT GOLDSTEIN (introduced by H. DAVIS). Central Institute for the Deaf, St. Louis, Mo.

Two electrical potentials have been associated with the cochlear response to sound: the action potential, a function of the neural element, and the cochlear microphonic associated with the structures of the organ of Corti, most likely the hair cells. Within the past two years 'rectification' of responses upon death of the animal has been observed, that is, the remaining responses are displaced to such an extent that they are predominantly or entirely above the baseline (positive in scala tympani) or below the baseline (negative in scala vestibuli). The pattern is that of a ripple of the stimulating frequency superimposed on this displaced baseline. Recent use of a 'tone pip' has shown summation of the excitatory process and has led to the identification of a *summating potential* which appears much the same as the post-mortem 'rectification,' positive in scala tympani and negative in scala vestibuli. However it is plainly visible in the living guinea pig even in the presence of action potentials. Upon cessation of breathing of the guinea pig and with the disappearance of the action potential, the 'rectification' gradually reverses itself and becomes negative in scala tympani and positive in scala vestibuli. If the animal can be revived soon enough, the responses will return to their normal pattern. Upon further deterioration of the condition of the animal and death the responses again reverse themselves, passing through the point of symmetry and then 'rectifying' according to the customary post-mortem pattern, positive in scala tympani and negative in scala vestibuli. (This work was performed under Contract N6onr-272 between the Office of Naval Research and Central Institute for the Deaf.)

*Effect of nerve stimulation on divided renal function.*

WILLARD E. GOODWIN,\* A. PAGE HARRIS\* AND W. W. SCOTT. James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Md.

A study of the effect of nerve stimulation on divided

renal function in the dog and monkey is described. This includes a method for explantation of the divided trigone and later simultaneous collection of urine from each kidney. Stimuli used were: 1) ice water to leg, 2) tourniquet to leg, 3) faradic sciatic stimulation, 4) faradic splanchnic nerve stimulation, and 5) faradic renal nerve stimulation. After control periods, differential function studies were made by dropwise collection of urine from each kidney and measurement of glomerular filtration and tubular excretion after constant infusion of phenolsulfonphthalein and sodium thiosulfate or *p*-amino-hippurate and creatinine. The results of these investigations are presented.

*Effects of peripheral stimuli on divided renal function.*

WILLARD E. GOODWIN, A. PAGE HARRIS AND W. W. SCOTT. James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Md.

Effects of unilateral peripheral stimuli on divided renal function have been studied in the dog and monkey. Stimuli were ice water or tourniquet applied to one hind leg after control periods of urine collection. Urine was collected dropwise from each kidney separately after previous explanation of the divided trigone. Renal function was measured by excretion of sodium thiosulfate and PSP (during Nembutal anesthesia) in one set of experiments and by excretion of creatinine and PAH (with chloralase anesthesia) in another group. A constant infusion was used. Clearances were not done. It was assumed that the arterial blood reaching each kidney was the same. There was usually a bilateral decrease in renal function, during and after stimulation, thought to be due to adrenaline and pituitary anti-diuretic hormone effects. This was usually followed by an increase, thought to represent 'reactive hyperemia.' *There was an additional unexpected unilateral effect in which the renal function on the side homolateral to the peripheral stimulus was decreased earlier and to a greater extent than on the unstimulated control side.* This was not always consistent either in magnitude or in occurrence; however, it appeared to be significant. The decreases in glomerular and tubular function were usually parallel. However, in some experiments the glomerular function seemed more impaired than the tubular; and the ratio of creatinine/PAH decreased on the side of stimulation. These results were interpreted as demonstrating, in some cases, the existence of a unilateral effect of peripheral stimuli on renal function. The greater effect on the side of stimulation was thought to be probably due to a neurovascular mechanism mediated by sympathetic reflex pathways from the extremity to the blood vessels of the kidney. No evidence for or against 'shunts' was adduced.

*Metabolism of polyphosphate in reference to treatment of metal poisoning.* R. E. GOSSELIN,\* A. ROTHSTEIN, H. L. BERKE,\* G. J. MILLER\* AND R. C. MEIER.\*

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Polymers of phosphoric acid form stable soluble chelation compounds with most cations, especially polyvalent cations. For example, commercial sodium hexametaphosphate (Na-HMP) complexes  $\text{UO}_2^{++}$  about 300 x more tightly than does citrate (at pH 3.5), and  $\text{UO}_2\text{-HMP}$  is probably 10,000 x tighter than the calcium complex (Rothstein and Meier, 1950). On the expectation, suggested independently by H. C. Hodge (1950) and one of us (R. E. G.), that hexametaphosphate might prove efficacious in the treatment of metal poisoning, preliminary observations of its mammalian metabolism are presented. In rats, the cumulative lethal dose (i.v.) of Na-HMP (48 mg. P/kg.) is about  $\frac{1}{2}$  that of a mixed calcium salt (Ca-Na-HMP). The greater toxicity of Na-HMP is ascribed to an acute reduction in the plasma level of ionized calcium. After intravenous administration, polyphosphate is demonstrated in blood by a marked rise in the concentration of labile P. In rabbits, its volume of distribution is 10 to 13% of the body weight. It is absent from blood cells. Because of *in vivo* hydrolysis of the polymer, the serum concentration of orthophosphate also rises moderately. Except for the gastrointestinal tract (from which only hydrolyzed phosphate is absorbed), specific sites of *in vivo* hydrolysis have not been determined, but none occurs *in vitro* in fresh blood, serum, or urine. In rabbits, polyphosphate is cleared from the blood within 2 hours after intravenous administration and within 6 hours after intraperitoneal administration, whereupon urinary excretion of polymer ceases. Levels of orthophosphate in serum and urine may continue above normal for 24 hours. In rabbits, about 30% of the dose (i.v. or i.p.) is accountable as labile P in urine, while rats usually excrete only 15 to 20%, even with an optimal i.p. dose (100 mg. P/kg.). Given i.p. (rats) together with 1.0 mg. uranium/kg. as  $\text{UO}_2(\text{NO}_3)_2$ , Ca-Na-HMP suppresses the urinary excretion of U by more than 50%, for reasons not yet understood.

*An experimental and comparative study of renal interstitial pressure.* CARL W. GOTTSCHALK (introduced by EUGENE M. LANDIS). Dept. of Physiology, Harvard Med. School, Boston, Mass.

Renal interstitial pressure was determined directly by thrusting into the kidney glass micro-pipettes (O.D. 100 $\mu$ ) or hypodermic needles (gauge 26, with lateral holes), connected by a 3-cm. length of thick-walled plastic tubing to a silicone-lined glass capillary (bore 0.56 mm.) at kidney level. With a continuous column of heparinized Ringer's to the middle of the capillary, movements of the meniscus were followed microscopically to determine what pressure on the meniscus would prevent fluid from entering or leaving the pipette tip, i.e. renal interstitial pressure. The accuracy of volume measurement was 0.005 cu. mm. permitting most determinations of interstitial pressure, with mul-

tiple readings, to be made with injection of 0.1 cu. mm. fluid or much less. In 66 anesthetized rats, guinea pigs, rabbits and cats renal interstitial pressure averaged 10 mm. Hg and in eight dogs 16 mm. Hg. As the renal venous pressure was increased by progressively compressing the renal vein, interstitial pressure remained constant until the venous pressure approached the pre-existing interstitial pressure and then began to rise. At renal venous pressures above 20 mm. Hg the interstitial pressure exceeded venous pressure by less than 2 mm. Hg. Death reduced both pressures to 2 and 5 mm. Hg in smaller animals and dogs, respectively. Interstitial pressure was independent of arterial pressures between 40 and 140 mm. Hg. An increase in ureteral pressure increased the interstitial pressure apparently by compressing the intrarenal veins. Local external pressure on the kidney produced a correspondingly local increase in interstitial pressure. The kidney does not behave as a simple bag of fluid; pressures in one area are not transmitted undiminished throughout the kidney.

*Inhibition of radioactive phosphate uptake in erythrocytes.* D. R. H. GOURLEY (introduced by CHALMERS L. GEMMILL). Dept. of Pharmacology, Univ. of Virginia Med. School, Charlottesville.

Continuing the investigation of the uptake of phosphate ions (labeled with  $\text{P}^{32}$ ) by human erythrocytes (*J. Cell. & Comp. Physiol.* 35: 341, 1950), it has been found that the rate of phosphate uptake by erythrocytes *in vitro* is depressed by the addition of iodoacetic acid (IAA) or sodium fluoride (NaF). The addition of higher concentrations of NaF brings about what appears to be a two-phase uptake of  $\text{P}^{32}$ . The initial phase is a process of rapid influx, the rate of which is not affected by a drop in temperature from 37° to 15° C., while the later phase is similar to the depressed chemical mechanism observed in the presence of IAA. A maximum inhibition (to about 20-25% of the normal rate) is observed in the presence of  $5.0 \times 10^{-4}$  M IAA or  $2.0 \times 10^{-2}$  M NaF. Wilbrandt (*Pflüger's Arch. f. d. ges. Physiol.* 243: 519, 1940) has shown that the glycolytic system in human erythrocytes is completely inhibited by these concentrations of the inhibitors. A combination of IAA and NaF inhibits the  $\text{P}^{32}$  uptake to a greater degree than the maximum observed with either of the two alone but does not cause complete inhibition. It is inferred that enzymatic reactions other than the phosphorylating reactions of glycolysis are involved in the transfer of phosphate into erythrocytes.

*Influence of estradiol on resistance of gastric mucosa to alcohol.* RHODA GRANT AND A. C. IVY. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

To test the possible anti-ulcer properties of a urine extract, a method was previously devised to measure the resistance of the rat's gastric mucosa (*Federation*

*Proc.; Am. J. Physiol.* 159: 571, 1949). It consisted of grading in arbitrary units the gross damage caused by 40% to 50% ethyl alcohol fed by stomach tube under standard conditions. The statistically significant increase in mucosal resistance found in rats pre-treated with extract might have been due to small amounts of estrogens present in the extract. Therefore the test has been used again on rats pre-treated with estradiol. This, in aqueous suspension or in oil, was injected intramuscularly over periods ranging from 10 days to 10 weeks, in total amounts of 248 to 475  $\gamma$  (av. 41-82  $\gamma$ /week). The difference in degree of mucosal damage between 17 control rats and 17 pre-treated with 248 to 475  $\gamma$  estradiol over a 3 to 8 week period was statistically significant ( $t = 2.03$ ;  $P = 0.05$ ). In a series of 9 control and 10 pregnant rats the average degree of damage was less in the latter but for the numbers of animals used the difference was not significant. This was also the case for 5 control rats and 6 given 314  $\gamma$  estradiol over a 10-day period. From data on the reproducibility of the alcohol test it was found that the degree of damage for 18 control rats in the first experiments did not differ significantly from that produced under the standard conditions in 26 control rats the following year.

*Value of radio-sodium clearance in evaluation of peripheral circulation in man.* HAROLD D. GREEN, CREED F. McFALL, JR.,\* MICHAEL J. MOORE\* AND G. ERICK BELL, JR.\* Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

Using the Kety method, 5  $\mu$ c.  $\text{Na}^{24}$  were injected into the gastrocnemius muscle. The rate of removal of  $\text{Na}^{24}$ , recorded with a heavily shielded end window Geiger counter tube connected to a counting rate meter, is

$$\text{expressed as } K = \frac{\log C_{T_1} - \log C_{T_2}}{0.4343 (T_2 - T_1)}; \text{ where } C =$$

counting rate and  $T =$  time in minutes. *Result:* a) Sixteen normals,  $K = 0.042$ ,  $\sigma = 0.019$ ; b) eleven patients with peripheral vascular disease (PVD),  $K = 0.031$ ;  $\sigma = 0.010$ ; probability (for difference between a and b) = 0.023. Although the groups are significantly different many of the patients had resting K values within the normal range. In attempting to obtain maximal rates of flow the following were studied: 1) The effect of 5 minutes of arterial occlusion; during occlusion  $K = 0.004$ , during first few minutes after release  $K = 0.103$  then gradually returned to control value ( $K = 0.035$ ). 2) The effect of 5 minutes of exercise of the gastrocnemius muscle;  $K$  during exercise = 0.094,  $K$  returned rapidly to control values after cessation of exercise ( $K = 0.038$ ). 3) During administration of Etamon and Priscoline sufficient to cause marked cutaneous vasodilation;  $K$  value was slightly but not significantly reduced from the control value in both normals and patients with PVD, by these vasodilator drugs. *Conclusions:*  $K$  parallels muscle blood flow. The degree of organic occlusive disease cannot be satisfactorily esti-

mated from resting K values. Maximal muscle blood flow necessary for estimating the degree of functional impairment of the circulation cannot be obtained by vasodilator drugs but may be approximated by exercise or reactive hyperemia.

*Electrically recording differential pressure flowmeter (Demonstration).* HAROLD D. GREEN, ALFRED W. RICHARDSON,\* GWEN ROBERTS\* AND NANCY C. KESTER.\* Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

This newly developed blood flow measuring system consists of a flowmeter tube constructed of a rigid polystyrene 26 cm. long and of uniform 3-mm. bore. The pressure drop between the two ends of the tube is transmitted to the opposite sides of a Statham differential strain gage (P-63). The gage is not sensitive to pressures applied equally to both sides and therefore is not affected by arterial pressure. However, it is very sensitive to differential pressures created by flow through the flowmeter tube. The potentials generated as a result of the pressure differential are amplified with a Brush strain analyzer and recorded either with an Esterline-Angus or a Brush magnetic recorder. The differential pressure is linearly proportional to flow up to  $\pm 100$  ml/minute, and deviates only 9% at flows of  $\pm 200$  ml/minute. In view of the linearity of response the apparatus can be used to record the contour of pulsatile flows, or by suitable electrical integration to record the true mean of a pulsating flow. The presently available strain gage, when assembled with the flowmeter tube has a natural frequency of 20 cps. At attenuation 50, which gives good stability, the assembled system will give a full-scale deflection on an Esterline-Angus recorder with a differential pressure of 4.3 mm. Hg, and on a Brush magnetic recorder with a differential pressure of 8.7 mm. Hg. This corresponds to 83 ml/minute and 175 ml/minute, respectively of blood flow. Higher rates of flow can be recorded by using greater attenuation.

*Comparison of effect of acetylcholine and epinephrine on normal and denervated heart of unanesthetized dogs.*

RUVEN GREENBERG AND CHARLES B. LAMBETH.\* Dept. of Physiology, Univ. of Texas Med. Branch, Galveston.

The comparison of the effect of a dose of a drug given intravenously in the same dog before and after heart denervation permits the study of the effect of the drug on the myocardium and blood vessels in the presence of the pressor receptor mechanism and in its absence. In a normal dog: 1) A minimal dose of acetylcholine produced a transient depressor effect accompanied by an increased heart rate. 2) A large dose of acetylcholine produced a temporary heart arrest, followed by an increased heart rate for 10 to 15 seconds until the b.p. rose to above resting b.p., after which there was a slowing of heart rate to less than normal lasting for

several minutes. 3) A minimal effective dose of epinephrine produced a pressor effect lasting 1 to 3 seconds followed by a depressor effect during which there was a slightly increased heart rate lasting for 10 to 15 seconds. 4) A larger dose of epinephrine produced a pressor effect and a slowing of the heart rate which lasted for several minutes. In the same dog with the heart denervated (a chronic preparation): 1) The same minimal effective dose of acetylcholine produced a transient vasodepressor effect and no change in heart rate. 2) The same large dose of acetylcholine produced a temporary heart arrest, followed by a gradual rise in pressure to slightly above normal and an increase in heart rate which began later and was much greater than in the normal dog. The increased heart rate of as much as 100% above resting level lasted several minutes and decreased gradually. 3) The same minimal effective dose of epinephrine produced a transient pressor effect, no depressor effect, and no change in heart rate. 4) The same large dose of epinephrine produced a marked pressor effect accompanied by a marked increase in heart rate which lasted for several minutes.

*Auricular flutter studied in direct leads from the human heart.* FRANZ M. GROEDEL AND MAX MILLER. Cardiological Dept. of Beth David Hospital, New York City.

During commissurotomy, performed on a patient with rheumatic mitral stenosis and chronic impure auricular flutter fibrillation, it was possible to do a direct electrographic exploration of the operatively exposed left auricle and its appendage; either one standard lead and one semi-unipolar direct lead, or two semi-unipolar direct leads from different sites of the epicardium were taken. At different sites of the auricle and its appendage, the electrograms varied greatly as follows: either rapid, or sharply pointed, or slow waves, or scarcely visible, or even reduplicated P waves were recorded; their time of appearance differed at the various sites—asynchronously—but the P-P intervals, at all areas, were fairly identical—isorhythmic. At sites farther distant from the venae cavae, the P waves occurred earlier than at sites nearer to the cavae. These first direct investigations on fluttering human auricles proved that polyfocal stimulation must be present in auricular flutter. There is great diversity in the excitability of the various portions of the auricular wall and its appendage, with minimal excitability at some areas, and the stimuli do not occur simultaneously, while the refractory period for every portion of the wall is quite identical. In the reduplicated P waves, seen at different sites, one wave is intrinsic, the other extrinsic, and the latter may be induced earlier or later than the intrinsic wave.

*Secretin, pancreozymin, duocrocin and cholecystokinin content of commercial secretin preparations.* M. I.

GROSSMAN, C. C. WANG\* AND K. HARTIALA.\* Univ. of Illinois, Chicago.

Two preparations of secretin are now commercially available: Secretin Lilly (*L*) and Secretin Wyeth (*W*). Two separate lots of each preparation were tested. Secretin potency was determined by the method of Gershbein, Wang and Ivy and gave the following values: *L* 13.5 u/mg. and *W* 5.3 u/mg. Pancreozymin was detected by the method of Harper using Secretin *L* to establish the basal flow. Two mg. of *W* caused about a 100% increase in amylase output in the 15-minute period after intravenous injection. Doses of *L* as high as 10 mg. failed to raise the enzyme output above the basal level. In dogs with isolated pouches of the Brunner's glands area, intravenous injection of 10 mg. of either *L* or *W* raised the secretory rate from a basal level of 0.2 cc/hour to an average of 3.5 cc/hour after injection. More highly purified secretin preparations had no effect on Brunner's glands secretion. Both *L* and *W* contain traces of cholecystokinin, probably not sufficient to influence the results in clinical diagnostic secretin tests. It is suggested that the pancreozymin content of commercial secretin preparations be taken into account in the establishment of normal standards of response to these substances for clinical diagnostic tests.

*Studies on controlled venous resistance.* ARTHUR C. GUYTON. Dept. of Physiology and Biophysics, Univ. of Mississippi Med. School, University.

In 32 dogs, the blood flow from the great veins has been diverted through an external system before emptying into the right auricle. Venous resistance and venous pressure may be controlled, and blood flow is measured by a stromuhr. As venous resistance is progressively increased, the venous pressure peripheral to the resistance increases up to approximately 4.5 mm. Hg above normal while the auricular pressure falls to 3 mm. below normal, the cardiac output falls progressively to zero, and the arterial pressure falls to 4.5 mm. pressure—equal to that in the peripheral venous system. It was calculated that capillary pressure decreased because the effect of falling arterial pressure was greater than the effect of rising venous pressure. Hematocrit studies support this deduction, for hemodilution was regularly observed following the application of venous resistance. Considering the venous resistance of these studies comparable to cardiac resistance in heart failure, even infinite resistance acutely applied at the heart would be incapable of elevating peripheral venous pressure to the high values often observed. It appears that acute cardiac failure would first greatly increase the cardiac resistance, though the backward pressure would not become great because of greatly decreased flow. When the venous return should approach normal again because of increased blood volume resulting from kidney shutdown, the peripheral venous pressure would be multiplied by the increasing flow which would pass

through the cardiac resistance. Thus it appears that congestion in heart failure results from a combined backward-forward mechanism.

*Subtotal adrenalectomy in essential hypertension: postural blood pressure responses in relation to type of cortical replacement therapy.* JOSEPH H. HAFKENSCHIEL, WILLIAM A. JEFFERS, FRANCIS D. W. LUKENS, HAROLD A. ZINTEL, NELLY J. KEFFER AND CHARLES C. WOLFFERTH. Edward B. Robinette Foundation, Med. Clinic, Hospital of Univ. of Pennsylvania, The Cox Institute and Dept. of Surgery, School of Medicine, Univ. of Pennsylvania, Philadelphia.

The pressor response to various adrenal cortical substitutes was studied following subtotal adrenalectomy in a 33-year old female. Bilateral thoracolumbar sympathectomy with complete removal of the right adrenal gland had been carried out nine months previously without benefit. After removal of 90% of the left adrenal, the patient was maintained on aqueous adrenal cortical extract totalling 240 cc. for the first 3 postoperative days, and cortisone totalling 37 mg. for the next 2 days. The patient was then afebrile, eating, ambulatory, and in electrolyte balance. The study of blood pressure responses began on the 7th postoperative day, when she was able to tolerate 3 minutes of quiet standing for the postural test. She was tested twice daily during the period herein reported. The first period comprised 8 days with no substitution therapy. Average pressures were then  $145 \pm 16/111 \pm 8$ , supine, and  $115 \pm 8/93 \pm 18$ , erect. While receiving adrenal cortical extract (ACE) 26 cc. intramuscularly for 5 days, the blood pressure averaged  $140 \pm 4/112 \pm 3$ , supine, and  $100 \pm 14/81 \pm 11$ , erect. A fixed salt intake of 5 gm. and desoxycorticosterone (DCA) 5 mg. i.m. daily was maintained for 18 days. Supine pressures averaged  $156 \pm 13/113 \pm 8$ , and erect pressures  $124 \pm 15/101 \pm 15$ . Cortisone (E) 12½ mg. daily was then administered while she was on same salt intake the following 5 days. Average pressures were  $146 \pm 23/91 \pm 8$ , erect. While receiving no adrenal cortical substitution therapy, but with 5.0 gm. of salt daily for the final 88 days, the patient was in good health and in normal electrolyte balance. The average supine pressure was  $128 \pm 7/91 \pm 6$ , and erect  $99 \pm 9/76 \pm 10$ . Supine pressures during the initial period without treatment as well as in the ACE, DCA, and E periods were all significantly higher than in the last. Supine pressures in the DCA period were higher than in the E period ( $P < 0.01$ ). Average erect pressures in the DCA period were significantly higher than those in the last period, but not significantly higher than the pressures of the first period without therapy, or the periods on other adrenal cortex substances. These data suggest that although DCA and salt in the above dosage increased the pressure in this patient while supine, the elevation in pressure while erect was not great enough to conclude that DCA was the most pressor of the steroids studied.

(This work was supported in part by the U. S. Public Health Service, the Squibb Institute for Medical Research and the Sandoz Pharmaceuticals Company. Generous supplies of cortical extract and desoxycorticosterone were made available by the Upjohn and Ciba companies for this study.)

*New studies on cardiovascular effects of desoxycorticosterone acetate.* C. E. HALL AND O. HALL. Carter Physiological Lab., Univ. of Texas Med. Branch, Galveston.

Twenty pairs of female or castrate male rats weighing 55 to 75 gm. were united in parabiosis. One week later they were given a 1% solution of sodium chloride to drink and two 50-mg. pellets of crystalline desoxycorticosterone acetate were implanted subcutaneously into the right lateral aspect of the right-hand partner. In this way the tissues of the left animal were prevented from absorbing the hormone directly. The animals were sacrificed 30 to 40 days later. In 15 pairs hypertension developed only in the left-hand partner. Histologically, in 9 instances where the blood pressure exceeded 200 mm. Hg, cardiac hypertrophy and widespread vascular lesions consisting of nephrosclerosis, periarteritis nodosa of the mesenteric and pancreatic arterioles were prominent. Interstitial myocarditis and granulomatous proliferations resembling 'Aschoff' nodules were seen in the heart. The remaining 6 pairs developed hypertension of mild degree, 150 to 180 mm. Hg, during the time of treatment and in these only cardiac hypertrophy was observed. In 5 instances hypertension occurred only in the partner bearing DCA, i.e. the right-hand animal. Of these, 3 with a severe hypertension showed vascular lesions similar to those described; while two developed a mild hypertension and only cardiac hypertrophy. We believe that hypertension developing in right-hand rats reflects a poor vascular union. Six of the DCA-implanted rats bore kidneys which resembled 'endocrine kidneys,' characterized by marked shrinking of the cortex, reduction in size and number of glomeruli, tubular atrophy and obliteration of tubular lumina. (This study was supported by a grant from the American Heart Association.)

*Somatic afferent areas I and II of the dog's cerebral cortex.* TERESA PINTO HAMUY,\* R. B. BROMLEY AND C. N. WOOLSEY. Dept. of Physiology, School of Medicine, Johns Hopkins Univ., Baltimore, Md., and Dept. of Physiology, Med. School, Univ. of Wisconsin, Madison.

The somatic afferent areas of the cerebral cortex of the dog have been mapped by the evoked potential method in order to provide information essential for other studies involving ablation of these areas. It has been possible to show that the body surface is so represented in somatic area I that the animal appears to be lying with tail and postaxial leg on the medial wall



of the hemisphere; the back falls along the caudal margin of the area, toward the visual cortex, while ventrum and limb apices are directed rostralward. There is continuity of foci for trunk, neck and face, and the whole pattern is so arranged as to form a distorted but integrated figure of the actual animal. The arrangement thus is similar to that previously described for the rat (Woolsey and Lemessurier, *Federation Proc.*, 1948). Somatic area II of the dog, in contrast to that of other species studied, has been found to possess a high degree of somatotopic organization. The arrangement of the area may be indicated by stating that the body surface is represented in such a way that the back of the animal lies along the dorsal border of the insula, while the limbs and face extend upward over the cortex toward somatic area I, with snout most rostral and tail most caudal. The results reported for other species are probably incomplete and therefore should not be taken as evidence for poorer somatotopic organization of somatic area II in those species.

*A new method for measuring colloid osmotic pressure.*

ANDERS TYBJAERG HANSEN. Dept. of Medicine B, Rigshospitalet, Copenhagen, Denmark, and Dept. of Medicine, Duke Hospital, Durham, N. C.

The methods for measuring colloid osmotic pressure suffer from certain drawbacks which have prevented their general use. The measurement requires 1) too much time and/or 2) too large a sample. An evaluation of the different factors involved in the measuring procedure shows that an improvement requires 1) reduction of the thickness of the membrane, 2) reduction of its area and 3) increase of the sensitivity with which the volume displacement across the semipermeable membrane is detected. In order to fulfill these requirements the writer has applied his electric condenser manometer (see A. Tybjaerg Hansen, *Acta Physiol. Scand. Suppl.* 68, 1949) for the detection of the volume displacement across the membrane, instead of the optical methods hitherto used. The needle is replaced by a hollow brass cylinder the free end of which is covered by a rigidly supported semipermeable membrane. The sample is placed on the membrane and subjected to an adjustable (air) pressure. The manometer acts as a zero instrument indicating in terms of positive or negative pressure whether the solvent passes the membrane in one or the other direction. Zero pressure indicates equilibrium between the colloid osmotic forces and the adjustable extrinsic pressure. The membrane is made of collodion (2%) in ether-alcohol (mixture 3/1). Thickness when dry, 5 to 10 microns. Its effective area is about 20 mm.<sup>2</sup> The sample required is less than 20 cu. mm. One measurement is accomplished in about 3 minutes, and subsequent measurements may follow immediately. The accuracy is about  $\pm 0.5$  cm. of water. The procedure is so that the actual reference liquid is the ultrafiltrate

of the sample. No thermostatic temperature control is needed. The molecular weight as calculated from measurements of samples containing pure ox serum albumin is about 67,000.

*Measurement of glomerular filtration in the dog: renal excretion of inulin, exogenous and endogenous creatinine and of sodium ferrocyanide.* KENDRICK HARE, RUTH S. HARE AND GEORGE S. HUSSON. Children's Hospital and Dept. of Pediatrics, Univ. of Buffalo, Buffalo, N. Y.

The validity of endogenous creatinine clearance as a measure of glomerular filtration rate in the dog was established by comparing this clearance with that of inulin and of sodium ferrocyanide in 50 simultaneous clearance periods in 4 adult female dogs. Inulin and ferrocyanide were given by constant intravenous infusion or by subcutaneous injection to produce a plateau of plasma concentration. All calculations were based on arterial bloods as the concentration of endogenous creatinine was invariably higher in peripheral venous than in arterial blood, especially if venous drainage of muscle was the source of the sample. Exogenous creatinine, which determined the method applied to endogenous creatinine, also had the same renal clearance as inulin and ferrocyanide.

*Osmotic diuresis in normal and diabetes insipidus dog.*

RUTH S. HARE, KENDRICK HARE, JULIUS COHEN AND JOEL WILLIAMS. Children's Hospital, Buffalo, N. Y.

Osmotic pressures of serum and urine were measured during osmotic diuresis produced by the infusion of 18% mannitol, 19% urea or 3% NaCl. In both normal and diabetes insipidus dogs a nearly linear relationship was found between the percentage of water reabsorbed and the percentage of solute reabsorbed, but at every point the diabetes insipidus dog reabsorbed less water than the normal. During the periods of maximal diuresis the diabetes insipidus dog excretes 15% more of the water filtered than does the normal; therefore, it may be assumed that during the control periods, all of the solute and 85% of the water are reabsorbed independently of Pitressin; this concentration reabsorbed may be calculated. In 14 control periods it averaged 1.15 times the serum osmotic pressure. If the same ratio of concentrations is applied to the periods during osmotic diuresis, the total water reabsorbed may be divided into a fraction associated with solute and a remainder. In the normal dog the extra water excreted is entirely derived from the first fraction; the remainder shows no change. In the diabetes insipidus dogs extra water is excreted from both fractions, the remainder often being completely excreted. It is concluded that only the second fraction of about 15% is under the control of Pitressin.

*Preparation of enterocrinin.* F. W. HEGGENESS\* AND

E. S. NASSET: Dept. of Physiology and Vital Economics, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Intestinal mucosa of swine is extracted with acidified ethanol and the extract concentrated *in vacuo* until the residue is a thick syrup. This residue is extracted 3 times with absolute methanol and the extracts combined and concentrated as before. The methanol-soluble material is dissolved in water and the enterocrinin precipitated as the flavianate. This precipitate is dissolved in alkali and flavianate ion removed as the barium salt. The active substance is then precipitated as the phosphotungstate, dissolved in alkali and phosphotungstate ion removed as the barium salt. Enterocrinin is extracted from the alkaline solution with butanol and adsorbed on cotton succinate from which it is eluted with dilute alkali. Flavianic acid added to this eluate brings down a crystalline product which decomposes sharply at 251°C. This product in doses of 0.4 to 0.8 mg. stimulates intestinal secretion in dogs.

*Effect of acid-base changes on flicker fusion frequency.*

CHARLES D. HENDLEY AND MATHEW ALPERN. Dept. of Physiology and School of Optometry, Ohio State Univ., Columbus.

Measurements were made of the critical flicker frequency (c.f.f.) under conditions of metabolic and respiratory acidosis and alkalosis at a variety of luminance levels on 13 normal humans. Conditions of mild metabolic acidosis (fixed acid = 7.2 mm/l.;  $\Delta pH = -0.11$ ) and alkalosis (fixed base = 6.9 mm/l.;  $\Delta pH = +0.11$ ) did not cause any significant variation in c.f.f. Conditions of respiratory acidosis ( $\Delta pCO_2 = +25$  mm. Hg;  $\Delta pH = -0.16$ ) and alkalosis ( $\Delta pCO_2 = -25$  mm. Hg;  $\Delta pH = +0.20$ ), on the other hand, showed highly significant changes in c.f.f. Respiratory acidosis produced by breathing a mixture of 7%  $CO_2$  and 93%  $O_2$  was accompanied by a fall in c.f.f. of approximately 4 cps at the 2 luminance levels measured (400 and 4 ml.). Respiratory alkalosis produced by hyperventilation was accompanied by a rise in c.f.f. of about 3 cps at all the luminance levels measured (4500, 400, 4, and 0.25 ml.). On the basis of the effects of anoxia on brightness discrimination and other visual functions, and of respiratory acid-base changes on the absolute threshold of the rods, this effect might have been expected only at the middle and lower luminance levels. These data suggest that changes in the  $pCO_2$  rather than pH changes per se affect the excitability of the nerve cells in the visual mechanism related to flicker discrimination.

*Effect of rate on cardiac excitability.* BRIAN F. HOFFMAN, JEROME L. GILBERT, ARTHUR A. SIEBENS, E. E. SUCKLING AND CHANDLER McC. BROOKS. State Univ. of New York College of Medicine, Brooklyn. The effect of heart rate on cardiac excitability has

been studied in dogs anesthetized with sodium pentobarbital (Nembutal). The exposed hearts were driven at 100 to 300 beats per minute and strength-interval curves established for each rate. The test shock was a square wave of 0.01 to 30 ma., positioned with respect to the driving impulse at any desired interval of the cycle. Test-shock durations were 0.1, 1.0, and 3.0 msec. Increasing heart rate shifts the ventricular strength-interval curve progressively to the left. This reflects a linear decrease in the duration of the total and absolute refractory periods without any change either in the relative refractory period or the diastolic threshold. Thus, while recovery starts earlier at faster rates, complete restoration of excitability occupies a constant amount of time. The effects of changes of rate on auricular excitability are similar to those in the ventricle. Shortening of the Q-T interval is approximately a linear function of rate in the range 100 to 300 beats per minute and parallels the changes in the total and absolute refractory periods. At all rates, the diastolic boundary of both the total refractory period and the irresponsive period bears a constant close relationship to the apex of the T wave. This relationship adds to the significance of the T wave with respect to the recovery of ventricular excitability.

*Further observations on drinking induced in dogs by hypertonic saline solutions.* JOSEPH H. HOLMES. Dept. of Medicine, Univ. of Colorado School of Medicine, Denver.

In these experiments using 13 normal mongrel dogs ranging in weight from 8 to 20 kg. the injection of 20% NaCl was utilized as a stimulus for inducing a fairly constant drinking response in the same animal. Offering of various types of drinking solutions showed that the intake was 2 to 3 times that of water when the animal was offered .9% saline. With 1.8% urea the intake averaged 1.3 times that of water. With all other solutions such as .5% glucose, 1.3% sodium acetate, .9%  $MgCl_2$ , .85%  $NH_4Cl$ , .9%  $NaHCO_3$  and 1.15% sodium acetate, the intake was the same or slightly less than that observed with tap water. Altering the temperature of the water drunk did not appreciably alter the drinking response. When water is introduced into the stomach prior to 20% NaCl it inhibits markedly the drinking response. This inhibition is not as great when 5% glucose, 1.8% urea or .9% NaCl are introduced into the stomach instead of water, but it is interesting to note that there was no great difference between the inhibition produced by .9% NaCl and that produced by 5% glucose or 1.8% urea even though the intakes when these substances were offered as drinking solutions were quite different. In contrast, intravenous infusion of like amounts of these same solutions had no inhibitory effect on drinking even though the hemodilution was more marked. Further, the drinking following either the intravenous infusion of 1.8% urea, .9% NaCl or 5% glucose did not differ markedly even

though the distribution of these various solutions in the body is quite different. The gastric factor, osmotic factors, and blood changes will be discussed.

*Nitrogen fractions as criterion of preservation of corneal tissue.* AUDREY HOWARD AND LUCY SIEGEL (introduced by WILHELM BUSCHKE). Ayer Foundation Ophthalmic Research Lab., Manhattan Eye, Ear and Throat Hospital, New York City.

Quantitative data on nonprotein nitrogen fractions in the tissue may be expected to furnish useful criteria for the state of preservation of corneal tissue stored under various conditions as donor material for transplantation. The present report deals with data on total nonprotein nitrogen, ammonia-nitrogen, and amino acid nitrogen which were obtained from beef corneas prior to and following incubation at 40°C. for 6 hours. Data were obtained from fresh corneas, from corneas stored in the whole eye in a moist chamber at 4°C., and from corneas stored under mineral oil or in an atmosphere over  $\frac{1}{2}$  molar and 1 molar sodium chloride solutions. As previously reported (BUSCHKE, W. XIX. Scientific Meeting, Association for Research in Ophthalmology, San Francisco, June 1950.), the cornea becomes progressively hydrated if stored in the whole eye. Following 3 days of cold storage (4°C), NPN and ammonia-N values were not significantly higher than those in fresh corneas. Likewise, freshly excised corneas incubated at 40°C. for 6 hours had NPN and ammonia-N values not significantly higher than fresh control corneas. Significantly higher NPN and ammonia-N values were found, however, in corneas which, following 3 days of cold storage in the whole eye, had been incubated at 40°C. for 6 hours. In contrast, corneas cold stored under conditions preventing hydration of the tissue, showed no or much smaller increases in nitrogen fractions on subsequent incubation at high temperature. The amino acid-nitrogen values fluctuated much under the various conditions, and no characteristic changes could be established for any one group. The observations are compatible with the notion that progressive hydration during cold storage conditions the tissue to autolytic processes or favors nitrogen catabolism at higher temperatures.

*Simultaneous multiple tissue clearances in measurement of trans-capillary diffusion rates.* CHESTER HYMAN, SAMUEL I. RAPAPORT AND RITA PALDINO. Dept. of Physiology, Univ. of Southern California, Los Angeles, and Radio Isotope Unit, Veterans Administration Hospital, Long Beach, Calif.

Stone *et al.* (*Proc. Soc. Exper. Biol. & Med.*, 1949) showed that the rate of venous appearance of an intra-muscularly injected material is a function of blood flow in the area and a factor dependent on the trans-capillary diffusion rate of the solute. Since blood-flow modifies the clearance rate, a direct evaluation of the trans-capillary diffusion becomes impossible.

This difficulty may be resolved in part by the injection of a mixture of two distinguishable substances. Here, the differences in the rate of clearance must be referable to differences in trans-capillary transfer since the blood flow is identical for both materials. A single injection of 0.2 to 0.5 ml. was made into the muscle of the forearm of human subjects or into the gastrocnemius of anesthetized rabbits. The injected material contained moderate quantities (2-5  $\mu\text{C}$ .) of each of two radioactive materials or one isotopic and one other substance. Blood samples were taken from the nearest large vein draining the site of injection. An aliquot of the injected material was diluted and used as a standard for comparison. The ratio of the concentration of the two injected materials was used in expressing the results of the experiment. In man, studies covering 15 minutes or less after the time of injection showed a relatively constant venous ratio of  $^{24}\text{Na}^+$  to  $^{131}\text{I}^-$  which was significantly higher than the standard injected ratio. A tendency of the  $^{131}\text{I}^-$  to  $^{32}\text{PO}_4^-$  ratio to fall with time was noted in experiments which lasted 45 to 60 minutes. The initial ratios were higher than the injected standard and fell in an approximately exponential fashion during the entire period. Similar experiments with  $^{131}\text{I}^-$  and  $\text{SCN}^-$  showed qualitatively similar results.

*Studies on blood flow in normal, denervated and spastic limbs of dogs.* C. J. IMIG,\* BARBARA FEUCHT RANDALL\* AND H. M. HINES. Dept. of Physiology, State Univ. of Iowa, Iowa City.

Determinations on the blood flow through the femoral artery of dogs were made by the use of an electromagnetic blood flow meter with the aid of either local or general anesthesia. Spastic limbs were produced by local injection of tetanus toxin. Denervation was accomplished by section of the sciatic and femoral nerves. Slow intravenous infusions of Priscol, Nor-epinephrine and Procaine caused an increase in blood flow. Etamon administration proved to be ineffective. The application of hot fomentations was followed by an increased blood flow through normal, spastic and denervated limbs. In anesthetized animals the greatest increase occurred in normal limbs; whereas in unanesthetized animals the greatest increase was found in denervated limbs. Passive stretching through a full arc of motion increased the blood flow through normal and spastic limbs. It proved to be most effective in the latter and was found to be ineffective in denervated limbs. Rhythmical electrical stimulation of normal muscles was accompanied and followed by an increase in blood flow.

*An electromagnetic hydraulic transducer for studying response characteristics of manometer systems to square wave and sine wave pressure variations of variable frequency.* JAMES ISAACSON AND RICHARD E. JONES

(introduced by EARL H. WOOD). Mayo Clinic, Rochester, Minn.

The device consists of a water-filled cylindric lucite chamber (dimensions: i.d., 1 inch; o.d., 2 inches; length, 1 inch) with incorporated fittings for introduction of needles and the like from manometer systems being tested. An electromagnetic drive unit is coupled to the chamber, through a circular  $1 \times 0.005$ -inch brass diaphragm. A variable frequency (0.5–50,000 cps) commercially available electronic oscillator actuates the drive unit, which translates the sine-wave oscillator current into sine-wave pressure variations in the chamber. The generated pressure variations are monitored by means of an unbonded strain-gage unit coupled directly to a similar brass membrane covering the opposite end of the pressure chamber. Constant-pressure amplitude at varying frequencies is achieved by maintenance of a constant current to the drive unit. This method is limited to the frequencies at which the mechanical resonance of the liquid system becomes effective. The resonant frequency of this pressure assembly without a test manometer attached is in excess of 1000 cps. Monitoring the output of a pressure-sensitive ceramic crystal located within the pressure chamber makes possible maintenance of constant-pressure amplitudes independent of resonance effects. Square-wave pressure variations are produced by making and breaking a D. C. current source to the electromagnetic driver. Time-delay characteristics can be determined by simultaneous recordings at suitable chart speeds of the test manometer trace and either monitoring strain gage or ceramic crystal or the trace of a suitable galvanometer in the drive-unit circuit proper.

*Calorimetry of rats immersed in cool water.* FRANK H. JACOBSON (introduced by E. F. ADOLPH). Univ. of Rochester, Rochester, N. Y.

The regulation of heat exchanges has been studied in the hypothermic rat. Fasted rats were immersed to the neck in a water-bath calorimeter initially at 20°C. The rates of heat loss and heat production were measured simultaneously. Colonic temperature declined rapidly to within approximately one degree of water temperature. The severe cold stress permitted only a brief initial increase of heat production. The rates of heat loss changed linearly with the temperature gradients, which suggests that the fraction of the heat loss carried to the surface by the blood was essentially constant. Although unpredicted by theory, a linear relation of colonic temperature and temperature gradient was found. Thus the approximate rate of heat loss could be calculated from colonic temperature alone. The overall transmission constant for heat loss is 74 calories/degree (C.) of gradient, or 82 cal/degree of colonic temperature. An effective 'thermal conductivity coefficient,'  $0.0014 \text{ cal/cm}^2/\text{sec}/^\circ\text{C}/\text{cm}$ , has been calculated by assuming the rat to be a cylinder in the steady

state, and producing heat uniformly throughout. This coefficient actually combines the effects of conduction, and of vascular convection. It appears, therefore, that physical regulation may be the animal's only significant defense against severe chilling since a regulation of heat production is inhibited by low temperature.

*Phreno-phrenic anastomosis in the dog.* N. C. JEFFERSON, C. W. PHILLIPS AND H. NECHELES. Dept. of Gastro-Intestinal Research, Med. Research Institute of Michael-Reese Hospital, Chicago, Ill.

The denervated diaphragm of the dog did not resume its respiratory function within a period of 20 months. At that time the musculature of the diaphragm had degenerated completely. Thus, anastomosis of a cut phrenic nerve or regeneration of a crushed phrenic nerve, must occur before 20 months. This was tested in dogs. The left diaphragm was capable of complete reactivation 3 months after complete section and primary anastomosis of the left phrenic nerve. Intercoastal and sympathetic innervation apparently cannot maintain the integrity of the diaphragm.

*Membrane potential and excitability of muscle fibers.*

H. P. JENERICK AND R. W. GERARD. Univ. of Chicago, Chicago, Ill.

If excitation results from depolarization of the membrane to some critical level, threshold should vary with membrane potential. Both measures were made on single frog sartorius fibers impaled with a microelectrode. Increasing external K lowers both linearly until the potential reaches 50 mv., when conducted responses are replaced by local ones. Potential and threshold at a given K are raised by increased external Ca, lowered by decreased Ca; but over a 30-fold range in Ca and a 5-fold range in K threshold parallels potential as this is varied. On the other hand, after shifting from one salt solution to another, the potential or the threshold change may be delayed by 10 minutes beyond the other; and even in flowing solution of constant composition, threshold may fall sharply after 30 minutes while potential is constant. Further, potential is alike in summer and winter frogs but threshold one-third less in summer. Ryanodine lowers potential with constant threshold, ACh, if anything, raises threshold at unchanged potential. Finally, potential is lowered by cooling ( $Q_{10} = 1.003$ ) while threshold is raised ( $Q_{10} = -1.7$ ). The membrane potential is, therefore, at most, one factor determining threshold. It seems more closely related to propagation of excitation.

*Intrahepatic lymphatics.* LEROY E. JOHNSON\* AND FRANK C. MANN. American Veterinary Association, and the Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

The liver is one of the largest sources of lymph in the body. The exact site of origin of hepatic lymph has

not been conclusively demonstrated. In an attempt to determine the possible physiologic significance of the liver in the formation of lymph, a comprehensive study of the intrahepatic lymphatic system was made. Observations were made concerning many individuals of 5 different genera: rats, guinea pigs, rabbits, swine and dogs. The method of investigation consisted mainly in retrograde injections of various media with a subsequent making of histologic sections and corrosive casts of the injected material. Microscopic examination of the slides reveals an intimate association existing between the lymphatics and all the other important anatomic components of the liver. Photographic evidence of these relationships was obtained and it depicts the hepatic lymphatics in their position relative to the components of the hepatic circulation and, to some extent, the hepatic cells. Of special significance is the relationship of lymphatics to the biliary system. By the use of multicolored plastics, points of possible communication were found between the two systems. In addition, the presence of lymphatics within the walls of the bile ducts, from which submucous radicles extend, was noted microscopically. These findings offer a possible explanation of some of the phenomena associated with the formation of hepatic lymph, pathogenesis of infections of the gallbladder and bile ducts, and so forth.

*Relation of cortisone, ACTH and DCA to metabolism of glucosamine and amino purines in synovialis of the dog.* NORMAN R. JOSEPH AND C. I. REED. Univ. of Illinois College of Medicine, Chicago.

The following amines were examined with reference to their effects on synovial membrane potentials of dogs: glucosamine, adenine, guanine, PABA and PASA. In isotonic Ringer solution all showed high positive potentials. Much smaller potentials were produced by the related non-amino compounds and by alpha amino acids. Mixtures of the above amines and monosodium glutamate in equimolecular concentrations also yielded small potentials. After intramuscular injection of 10 mg. of cortisone only small potentials were produced by glucosamine, PABA and PASA. Adenine and guanine potentials were only slightly decreased by 10 mg. of cortisone, but were diminished by about half when 20 mg. were injected. Injection of ACTH and DCA also modified the reactions obtained with glucosamine, PABA and PASA.

The modification by cortisone of the reaction with glucosamine is interpreted as an effect on deamination of transamination reactions, analogous to that produced by glutamate.

*Litten's phenomenon in a normal subject* (Motion picture). FREDERIC T. JUNG, MADGE WALSH AND ROBERT J. WIESE. American Medical Association, Chicago, Ill.

Litten's phenomenon is the movement of a shadow

seen on the sides of the chest as the diaphragm descends during inspiration. It is seen best in sparely built people under proper illumination during deep diaphragmatic breathing. The student shown in this film not only displayed the phenomenon unusually well, but was also able to alternate between deep diaphragmatic and deep costal breathing at will. It is explained by the fact that the intercostal spaces tend to be flat wherever the liver presses through the diaphragm against the chest-wall, but tend to sink in when the liver is pushed away by the diaphragm descending during inspiration. On watching it, one can imagine seeing the liver through the body-wall. This phenomenon is instructive to students of physiology because 1) it shows the amplitude of the movements of the liver during respiration, 2) it explains much of the pain in pleuritis and peritonitis, and 3) it explains why thoracentesis too near the costal margin may lacerate the liver.

*Effect of carcinogens on regeneration of forelimbs of larval salamanders.* ALEXANDER G. KARCZMAR AND GEORGE G. BERG (introduced by THEODORE KOPANYI). Pharmacology Dept., Georgetown Univ. Med. Center, Washington, D.C.

The regeneration of amputated forelimbs was followed during the span of normal regeneration (26 days at 20°C.) in 3 series of *Amblystoma punctatum* larvae: 1) kept in water solutions of 1,2,5,6-dibenzanthracene 9,10-endo $\alpha$ , $\beta$ -succinate (DBAS) of increasing strength (0.2-40.0 mg. %; 32 larvae); control animals were exposed to similar concentrations of desoxycholate or reared in pond water (30 larvae); 2) exposed to 20 to 40 mg. % of DBAS for brief time intervals prior to, or simultaneously with, amputation and returned thereafter to pond water (20 larvae); injected subcutaneously in the vicinity of the forelimb with 20-methylcholanthrene in olive oil (1%, 9 larvae) or with olive oil alone (controls, 8 larvae). The growth inhibition which resulted was first noticeable, in series 1, in larvae exposed to 2.0 mg. % and depended thereafter on concentration of DBAS; complete growth inhibition and some regression of the amputated limb occurred with strongest concentrations. The inhibitory effect of injected methylcholanthrene was comparable to that of 16 mg. water solution of DBAS. Briefer DBAS treatments were also effective, regeneration rates not returning to normal after 45 days in pond water. Inhibition of regeneration by carcinogens, controversial in the case of amputated forelimbs of adult urodeles, is thus confirmed and supports Haddow's (*J. Path. & Bact.* 47: 567, 1938) data on their inhibition of neoplastic growth. (This research was supported by a grant from the Cancer Institute of the Public Health Service.)

*Comparison of Ringer diuresis and water diuresis in normal and adrenalectomized rats.* RALPH H. KELLOGG

AND W. RICHARD BURACK (introduced by E. M. LANDIS). Dept. of Physiology, Harvard Med. School, Boston, Mass.

A method has been developed for measuring water and electrolyte balances in unanesthetized adult male rats over a period of several days during which nourishment is maintained by a constant flow of evaporated milk solution through a gastric catheter. Insensible weight loss and exact urination times are indicated by use of an electrically recording balance. Because of our interest in the regulatory mechanisms normally controlling extracellular fluid volume, we have been studying the diuresis produced when extracellular fluid volume is expanded by the oral administration of an isotonic saline fluid such as Ringer's solution. Studies have included the effects on this 'steady state' preparation of suddenly injecting 5 ml. of distilled water or 5 ml. of Ringer's solution into the gastric catheter, while the flow of milk continues. The resulting water and Ringer diureses have been compared with diureses similarly induced in adrenalectomized rats maintained with added salt. Within a few days following adrenalectomy, rats lost the ability to have a water diuresis under these circumstances, as was expected from the more extensive work of Gaunt and others. Seven experiments in 4 adrenalectomized rats showed that the diuretic response to Ringer's solution, including the associated increase in salt excretion, may still take place after the diuretic response to water fails. The observations indicate that when the extracellular fluid volume of a rat is expanded by the administration of Ringer's solution in this way, the excretion of the excess water, sodium, and chloride may be independent of the adrenal gland.

*Physiological effects of administration of oxygen under positive pressures to anesthetized dogs at low barometric pressures.* JOHN KEMPH AND FRED A. HITCHCOCK. Lab. of Aviation Physiology, Ohio State Univ., Columbus.

Dogs exposed to 30 mm. Hg ambient pressure for 3 minutes and given oxygen at intermittent positive pressure show a 50% mortality. Evidence obtained from arterial oxygen saturation, X-rays and autopsies indicates that the cause of death is stagnation anoxia resulting from aeroembolism. If the thorax and abdomen are bound and 90 mm. Hg intrapulmonic pressure is administered intermittently, survival time at 30 mm. Hg is greatly increased. Twenty dogs have survived exposures of 10 to 120 minutes. Postexperimental observation revealed no apparent abnormality. Arterial blood samples taken while the dogs were at 30 mm. Hg showed a very low total CO<sub>2</sub> content, an increased hematocrit over control and arterial oxygen saturation of 25 to 70% depending upon how the animal was respired. When intrapulmonic pressure was given there was a transient increase in the mean blood pressure followed by a gradual decrease, and a

gradual diminution in pulse pressure. Release of intrapulmonic pressure resulted in a sharp drop in mean blood pressure followed by a rapid rise to normal. High intrapulmonic pressures were tolerated better at 30 mm. Hg than at ground level. X-ray studies show that high intrapulmonic pressure increased the thoracic cage in transverse and vertical diameters with very little effect on heart size. It is believed that high positive intrapulmonic pressures with restraint maintained the life of anesthetized dogs exposed to 30 mm. Hg ambient pressure by preventing intracardiac and intravascular gas bubble formation and by decreasing the degree of anoxic anoxia. (This research was done under contract with the Aero-Medical Laboratory, Wright-Patterson Air Base, Dayton, Ohio.)

*X-rays on explosively decompressed dogs.* JOHN P. KEMPH, EDWIN G. VAIL, BUFORD H. BURCH, DONALD ROSENBAUM, S. A. FRYE AND FRED A. HITCHCOCK. Lab. of Aviation Physiology and Medicine, Ohio State Univ., Columbus.

Many of the conventional methods used in physiological research cannot be used at ambient pressures below the vapor pressure of the body fluids because of the release of dissolved gases from fluids and the vaporization of fluids. X-ray studies have been very useful in determining the physiological effects of exposure to these low pressures. X-ray films taken in these studies have been demonstrated. Most of the body cavities including the heart and interpleural space show distention from expanding gases and vaporization of fluids. Great subcutaneous swelling occurs. The time at which circulation stops has been demonstrated to be usually within 20 seconds after decompression. X-ray studies of the effects of binding the thorax and abdomen and applying positive intrapulmonic pressures were also demonstrated.

*Factors influencing output of bile acids in dogs.* K. S. KIM, D. F. MAGEE AND A. C. IVY. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

For this study we have used chronic external biliary fistula dogs. For the total cholate determination, we have used the method described by Irvin *et al.* (*J. Biol. Chem.* 153: 439, 1944). To determine endogenous cholate formation we fasted 4 dogs from 6 to 8 days without returning bile. It is estimated to be about  $100 \pm 23$  mg/kg. body weight in 24 hours. Protein has been shown to increase the output of cholate in chronic biliary fistula dogs. We attempted therefore to determine whether a mixture of pure amino acids could increase the cholate output. We have found that supplements of amino acid solution containing 29 gm. of the D-L forms of the 10 essential amino acids plus 23 gm. of glycine evoked a highly significant increase (average 730 mg.) in the daily output of cholate (12 tests on 10 dogs). A mixture of the ketogenic essential

amino acids increased cholate output, but not as much as with a complete mixture. The glycogenic and those failing to fall in either group showed no effect. Cortisone, 50-100 mg., was without effect (4 tests on 4 dogs). Vitamin B<sub>12</sub>, 7.5 µgm., increased the cholate output for the first 24 hours only. On following days even continuing the B<sub>12</sub> the cholate often returned to or below its original level (8 tests on 5 dogs).

*Thyroxine-inhibiting effects of iodinated phenoxyacetic acids.* H. M. KLITGAARD,\* H. B. DIRKS, JR.\* AND S. B. BARKER. Dept. of Physiology, State Univ. of Iowa, Iowa City.

A nearly complete series of iodinated phenoxyacetic acids was tested for thyroxine-inhibiting action in thyroidectomized male rats maintained on 12 µgm. of thyroxine/kg/day. The inhibitor was usually given at a ratio of 500 molecules of inhibitor to one of thyroxine and oxygen consumption changes studied. 2-Monoiodophenoxyacetic acid was found to have 21.0% inhibition, while 3-monoiodophenoxyacetic acid showed an inhibitory effect of 56.0%. Inhibition of 42% was found when 4-monoiodophenoxyacetic acid was administered. 2,4-Diiodophenoxyacetic acid at the usual dose showed inhibitory activity of 46.0%, while 2,3-diiodophenoxyacetic acid and 6-methyl-2,4-diiodophenoxyacetic acid were found to have no inhibitory effect. No anti-thyroxine activity was found upon testing 2,5-diiodophenoxyacetic acid, while 2,6-diiodophenoxyacetic acid showed marked inhibitory effect of 67.0%. 4-Methyl-2,6-diiodophenoxyacetic acid was found to have only 7.0% inhibition, and 2,4-diiodophenoxyacetic acid and 3,5-diiodophenoxyacetic acid showed no inhibitory activity at the usual dose level. The activity of 2,4,6-triiodophenoxyacetic acid exceeded any iodinated compound tested in this series with 81.0% inhibition, while only moderate antagonism of 32.0% was found in 5-methoxy-2,4,6-triiodophenoxyacetic acid. The concept is advanced that iodine in the 2, 4, and 6 positions on the phenyl ring of phenoxyacetic acid produces thyroxine-inhibiting properties.

*A new separation of several sets of pathologic mechanisms which occur during different kinds of circulatory 'shock,' any single one of which acting maximally can alone, by itself, cause peripheral vascular failure and death of subject.* M. H. KNISELY, S. BARKER, E. H. BLOCH, A. LIPSCOMBE, L. WARNER, F. BROOKS, L. R. DRAGSTEDT, C. L. SCHNEIDER, V. S. LEQUIRE, H. BERRINGTON STONER, AND JOHN IRWIN. Depts. of Preventive Medicine and Physiology, Univ. of Tennessee, Memphis; Depts. of Anatomy and Surgery, Univ. of Chicago, Chicago, Ill.; Herman Kiefer Hospital, Detroit, Mich.; Dept. of Anatomy, Vanderbilt Univ., Nashville, Tenn.; Dept. of Pathology, Univ. of Sheffield, Sheffield, England; Massachusetts General Hospital, Boston, Mass.; and Dept. of Anat-

omy, Med. College of State of South Carolina, Charleston.

Some kinds of shock can be separated experimentally. In the clinic, several kinds may be occurring simultaneously in one patient. If, and whenever, the whole circulatory system fails, some part or parts of the system must be behaving in such a fashion as to permit or cause the blood to stop circulating. Any such reaction should be detectable. The subjects presented are: 1) Endothelial leakage which can be caused by: a) anoxia; b) toxic substances. 2) Hemorrhagic shock without trauma. Human blood donors developed prolonged complete vessel spasms, but no sludge. Reopened anoxic vessels leak rapidly. 3) Reservoir retention shock, without hemorrhage or sludge. The outlet valves of the liver close tightly, forcing the great portal reservoir to store so much blood that failure of venous return occurs. (For basic anatomy and physiology, see KNISELY, BLOCH AND WARNER, 1948, and THOMAS AND ESSEX, 1949). 4) Sludge shock initiated experimentally without hemorrhage. Thick sludge forms wherever blood flows past crushed or burned tissue; the whole circulating blood changes to a stiff sludge causing reduced flow rates through small vessels all over the body. This causes stagnant anoxia of endothelium which results in plasma leakage and vessel plugging which cumulatively cause failure of venous return. 5) Spasm of pulmonary artery. The above have been observed. From autopsy reports on human anaphylactic death, it is also possible to deduce. 6) Spasm of veins between brain and thorax.

*Urinary sodium in guinea pigs on diets deficient in anti-stiffness factor.* HUGO KRUEGER, ROSALIND WULZEN, LLOYD YONCE AND PHILLIP LEVEQUE. Dept. of Zoology, Oregon State College, Corvallis.

Guinea pigs, on as adequate a diet as possible, and guinea pigs on diets deficient in the anti-stiffness factor (DASF guinea pigs) were placed singly in metabolism cages for 36 hours with water available but with no food. The urines were collected every 6 hours. For 20 36-hour experiments on 7 control guinea pigs, the urinary volumes ranged from 18 to 59 cc.; and for 20 experiments on 6 DASF guinea pigs, from 39 to 141 cc. The experiments began at 1800 and were completed at the second 600 following. This allowed comparison of a night period, a day period and a second night period. In most control guinea pigs the output of urine was higher the second night period than the first. The day output was higher than the output for either the preceding or succeeding night period. In the DASF guinea pigs, the output was very high during the first 6-hour period. The curves for the rate of urine output of DASF guinea pigs against time approximated rectangular hyperbolae with both asymptotes truncated. A pure hyperbolic form was modified in some experiments by data suggesting that in the DASF guinea pigs, as well as in the controls, factors were

present leading to a greater output during the second night than the first night and to a greater output during the day than at night; but the factors producing the very high initial rate of urinary excretion in the DASF guinea pigs tend to make the detection of less potent factors difficult. In guinea pigs on the better diet the rate of Na excretion was low initially, and then increased to reach a maximum either during the daylight period or the second night period. In DASF guinea pigs Na excretion started very high and subsequently decreased. Total Na in the urine for the 36-hour period ranged from 3 to 61 mg. in control guinea pigs and from 17 to 180 in the DASF guinea pigs. The average total Na excreted was 24 mg. in 36 hours for controls and 63 mg. in the DASF guinea pigs.

*Spontaneous activity of rats during dietary restriction and rehabilitation.* HARRY J. KRZYWICKI AND ESTHER DACOSTA (introduced by A. J. CARLSON). U. S. Army, Med. Nutrition Lab., Chicago, Ill.

Sixty male albino rats were divided into 6 groups of 10 each. In each group 4 rats were used as controls and 6 were placed on a carrot diet (2% protein) for 13 weeks. Two rats from each group were killed for analysis at this time and 4 were rehabilitated on either a high protein, a high fat, or a high carbohydrate diet for 13 weeks. Food consumption, body weight, and spontaneous activity were followed throughout the study. While on the carrot diet the average food intake decreased from 30 gm. to 18 gm./rat/day; spontaneous activity decreased approximately 25% below that at the beginning of restriction and a weight loss of about 40% was noted. During the early stage of rehabilitation, when rapid weight gains were occurring, activity fell below that observed towards the end of the restriction period. This was followed by an increase in activity, approaching the control values, in the rats rehabilitated on a high protein diet. On a high fat diet the activity equalled and in some cases exceeded that shown by the controls. The activity of the animals on the high carbohydrate diet approximated that of the controls. Although certain forms of restricted diets, reported previously, led to an increase in spontaneous activity, when the protein content of the diet is very low (2%) a marked decrease in spontaneous activity occurs. This may be due to changes in the endocrine system or an overall protein depletion.

*Growth hormone induced hypoglycemia in the depancreatized animal.* M. KURTZ,\* R. C. DE BODO, S. P. KIANG\* AND A. A. ANCOWITZ.\* Dept. of Pharmacology, New York Univ. College of Medicine, New York City.

Previous work has shown (*Federation Proc.* 9: 30, 1950; *Proc. Soc. Exper. Med.* 74: 524, 1950) that purified anterior pituitary growth hormone 1) exerts a very potent anti-insulin action in hypophysectomized dogs, i.e. it diminishes and, on continued administration,

abolishes the exaggerated hypoglycemic response to insulin; 2) it produces a concomitant diabetic state. In contrast to the anti-insulin action which was noted as early as 18 hours after the first injection of growth hormone the immediate effect of the first dose of growth hormone on blood sugar was a marked hypoglycemia provided that the growth hormone was given in the post-absorptive state. The degree and duration of the hypoglycemia was very similar to that seen following the administration of insulin. This effect was observed only after the first injection of growth hormone. When the growth hormone injection was repeated after 24 hours, again to animals in the post-absorptive state, no change in blood sugar occurred. Thus, the typical anti-insulin action of growth hormone already was present. In order to determine whether the Langerhans islets are involved in the growth hormone induced hypoglycemia, experiments were carried out on acutely depancreatized animals. In the untreated dogs the blood sugar showed a marked elevation following pancreatectomy. In contrast to this when growth hormone was administered to the acutely depancreatized dogs, a marked fall in blood sugar occurred. It is concluded that the pancreas is not essential for the development of the growth hormone induced hypoglycemia since it also occurs in depancreatized animals.

*Determination of respiratory dead space made by means of a mass spectrometer.* GEORGE H. KYDD, III (introduced by FRED A. HITCHCOCK). Lab. of Aviation Physiology and Medicine, Ohio State Univ., Columbus.

Work previously reported from this laboratory indicates that the ratio of oxygen absorbed to CO<sub>2</sub> given off during an expiration is not constant. This is contrary to the generally accepted idea of the constancy of alveolar air. It was decided to find what part of the dead space air as calculated by Haldane's and Bohr's methods could be attributed to the pure dead space portion and what part was contained in the mixed dead space-alveolar portion. This determination is facilitated by the use of the Mass Spectrometer in that the CO<sub>2</sub> can be detected in the expired air very soon after it reaches the inlet of the instrument. Thus by placing the inlet of the spectrometer just outside the subject's mouth and recording expired air flow with a flowmeter, one can determine the volume of air expired preceding the appearance of CO<sub>2</sub>. Then by calculating the dead space by another method, the volume of mixed alveolar and dead space air can be obtained. In three experiments the agreement was within 20% but the most significant finding is the fact that such a large portion of the dead space as calculated by Bohr's method is contained in this first portion of expired air even during hyperventilation. This is especially significant in view of our previous findings that the R.Q. of this gas is not constant. Results from the other series show dead space to be 163 normally, 410 during voluntary hyper-



ventilation and 312 during involuntary hyperventilation.

*Effect of epinephrine and other drugs on myotonia congenita.* EDWARD H. LAMBERT AND SIBYL BECKETT.\* Mayo Foundation and Mayo Clinic, Rochester, Minn.

The following test has been used to assay the action of drugs on myotonia congenita. The ulnar nerve at the elbow is stimulated with supramaximal shocks at a rate of 5/second for 30 seconds. Flexor tension of the 5th finger is recorded relatively isometrically by a resistance wire strain gage. The muscle-action potential is recorded by surface or needle electrodes. After 2 to 4 twitches myotonia develops, becoming maximal within 4 to 8 seconds. During this time spontaneous electrical activity occurs, while the twitch and action potential decrease in amplitude and reach a minimum within 6 to 10 seconds. With continued stimulation spontaneous activity ceases and myotonia decreases. Subsequently, the twitch and action potential increase, the twitch reaching 200% of its initial amplitude at 30 seconds, even though the action potential may still be less than its initial amplitude. Responses of this type are obtained uniformly when the interval between tests is 10 to 15 minutes. Epinephrine injected intravenously temporarily increased the initial twitch, greatly delayed the onset (0.06 mg.) or abolished (0.2 mg.) myotonia and reduced potentiation of the twitch. Its effects in many respects resembled those of exercise in reducing myotonia. Epinephrine was more effective intra-arterially than intravenously. Nor-epinephrine had little or no effect. Procaine given intra-arterially reduced the initial twitch and reduced and slightly delayed the onset of myotonia. Quinine had no effect on initial twitch, but reduced and slightly delayed the onset of myotonia. D-Tubocurarine given intra-arterially reduced the initial twitch, but had no effect on myotonia.

*Synthesis and biological activity of 6,7-diethyl-9-(D-1'-ribityl)-isoalloxazine, an analogue of riboflavin.* JOHN P. LAMBOOY (introduced by W. O. FENN). Dept. of Physiology and Vital Economics, School of Medicine and Dentistry, Univ. of Rochester, Rochester, N. Y.

The recent availability of o-diethylbenzene has made the synthesis of 6,7-diethyl-9-(D-1'-ribityl)-isoalloxazine possible. The compound differs from riboflavin, of which it is an analogue, by possessing two ethyl groups in the 6 and 7 positions instead of two methyl groups. The synthesis was accomplished by a series of reactions leading to 4,5-diethyl-2-nitroaniline which, when condensed with D-ribose and eventually with alloxan, produced the desired 6,7-diethyl-9-(D-1'-ribityl)-isoalloxazine, or Diethyl-riboflavin. Diethyl-riboflavin is very similar to riboflavin in all chemical and physical properties studied. At present the biological activity of this compound has been studied only on

*Lactobacillus casei*. Diethyl-riboflavin not only supports growth of this organism but is able to do so as the sole source of flavin. Its ability to support growth has been determined by the routine procedure for the assay of riboflavin. Equimolecular concentrations of riboflavin and diethyl-riboflavin were treated as independent standards and when compared on the basis of a 3-day growth period, they were indistinguishable in their ability to support growth. When the growth period was extended to 5 days at flavin concentrations which were limiting and also at levels which were non-limiting, it became evident that riboflavin was superior. The ability of diethyl-riboflavin to serve as the sole source of flavin was demonstrated by the ability of the compound to support growth not only in the first subculture but continuously throughout 6 successive serial subcultures.

*Physiological and clinical significance of a respiratory depressor reflex.* ALFRED LEIMDORFER. Dept. of Pharmacology, Loyola Univ. and Cook County Hospital, Chicago, Ill.

Some time ago, I reported that in respiratory arrest at the end of a maximal inspiration of normal persons a depressor reflex can be elicited (a fall in systolic blood pressure accompanied by a slowing of the heart and flattening or inversion of the T-wave, mainly in lead II of the electrocardiogram). The reverse of these changes occurs to a lesser extent in respiratory arrest at the end of a maximal expiration. This depressor reflex seems to be evoked by stimulation of sensory pressoreceptors in the pulmonary vascular bed during the great inflow of blood due to the forced inspiratory position of the chest. These findings are in conformity with the results of experiments of Schweig, Daly and Parin. They found in acute experiments on dogs that an increase in the pulmonary pressure was followed by a fall in systemic blood pressure and slowing of the heart. This reflex might be of importance for the proper distribution of blood to the lungs (i.e. during change in posture or during great muscular exercise). Further, it might play a rôle in protection of the lung from overflooding with blood under pathological conditions (i.e. during the rise in pulmonary pressure due to the impaired activity of the left heart or due to the increased work of the right heart). In patients with essential hypertension (of the labile type), this depressor reflex is exaggerated. However, hypertensives with fixed blood pressure fail to respond to this reflex. These facts might serve as additional tests for the selection of hypertensives for surgical treatment.

*An electronically weighted and stabilized central terminal for electrocardiography.* RAPHAEL B. LEVINE\* AND OTTO H. SCHMITT. Biophysics Group, Univ. of Minnesota, Minneapolis.

Electrocardiographic equipment as used in standard and laboratory practice is commonly susceptible to a large variety of distorting influences which interfere to

a greater or lesser extent with the recording of accurate electrocardiograms. Two of the most troublesome of these are those due to inaccuracy of the ordinary central terminal, and those due to interference from nearby wiring and from the recording equipment itself. An electronically stabilized central terminal has been developed using servo-amplifier techniques to eliminate or counteract these types of distortion. This improved central terminal is designed to reduce substantially electrostatic interference from wiring in the neighborhood of the patient, and to practically eliminate A. C. fed back from the standard recording machines into the patient. Because it has high input impedance, it reduces loss of signal due to high resistance leads, such as are often encountered in catheterization. The effect of electrode resistance on the accuracy of the central terminal is substantially eliminated. At the same time servo-grounding grounds the patient many times more effectively than does connecting a limb directly to earth. Provision is also made to use any desired number of electrodes and to assign different weights to each in determining the potential of the central terminal.

*Effect of acute reduction of cardiac output on clearance of sodium.* MATTHEW N. LEVY (introduced by CARL J. WIGGERS). Dept. of Physiology, Western Reserve Univ. Med. School, Cleveland, Ohio.

When cardiac output is acutely reduced in dogs by means of graded partial occlusion of the pulmonary artery a significant diminution in the clearance of sodium results. This impaired sodium excretion occurs under conditions of both normal and elevated plasma sodium levels, but the relative changes are much greater when the plasma sodium concentrations are within normal limits. The principal factor responsible for the decreased sodium clearance is the reduced glomerular filtration rate, which is in turn due to the diminished cardiac output. However, there is not an absolutely consistent correlation between the rate of sodium excretion and the sodium load presented to the tubules for reabsorption. Various factors may modify this relationship. For example, the plasma sodium concentration per se is shown to exert a powerful influence upon sodium elimination. There is no evidence in any of our experiments for the operation of a Tm mechanism for sodium reabsorption. Even at high plasma sodium concentrations the rates of sodium reabsorption change from control period to control period, and during occlusion periods the rates of sodium excretion are relatively high, even though sodium reabsorption is significantly reduced.

*Study of crossed phrenic phenomenon.* L. J. LEWIS AND JOHN M. BROOKHART. Dept. of Physiology, Univ. of Oregon Med. School, Portland.

Diaphragmatic activity of barbiturate anesthetized dogs and cats with spinal cord hemisections at C<sub>1</sub>-C<sub>2</sub> was studied during various experimental procedures.

Activity of each hemidiaphragm was indicated by cathode ray oscillograms recorded simultaneously over matched channels. In some experiments the amount of activity was quantitated by counting the number of single motor unit twitches during each inspiration. Activity over both direct (side of intact cord) and crossed (side of hemisection) pathways was present in the control state. Activity on the crossed side, as well as direct activity, could be increased by those maneuvers which increased central respiratory discharge and decreased by those maneuvers diminishing central respiratory discharge. Under spontaneous ventilation, increased crossed activity occurred after paralysis of the direct phrenic. Under controlled artificial ventilation, direct phrenic paralysis produced no detectable change in crossed activity. Similar results have been recorded from inspiratory internal intercostals. It is concluded that crossed respiratory activity is entirely independent of peripheral interruption of the direct phrenic pathway, but is directly proportional to the intensity of central respiratory discharge, a resultant of many factors.

*Cutaneous vascular responses in rabbit determined fluorometrically following local UV irradiation.* EDWARD A. LICHTER AND ALFRED A. SCHILLER. Dept. of Physiology, Univ. of Illinois College of Medicine, Chicago.

The depilated abdomens of rabbits were exposed to single, moderate and intense erythral intensities of UV irradiation over several 20-mm. diameter areas; contralateral filtered sites serving as controls. Photometric measurements of the intensity of cutaneous fluorescein fluorescence in these sites were made at 2- to 4-minute intervals for 60 minutes according to the procedure described by Schiller (*Proc. Soc. Exper. Biol. & Med.* 72: 594, 1949). Evidence indicates that during the first 20 minutes following the i.v. administration of fluorescein, transudation dominates, which is superceded by reabsorption in the last 40 minutes. Post irradiation dye measurements, of this type, in each of several animals, were made at frequent intervals up to 55 to 60 hours to provide a time pattern of the cutaneous vascular responses. Under comparable conditions, moderate UV irradiation shows, early, a slightly increased transudation (+15%) followed by a rapid decrease (-30%) beginning in 24 hours. The reabsorption is initially depressed (-60%) and progresses rapidly up to 24 hours (-150%), followed by an abrupt recovery during the next 24 hours. Intense UV irradiation causes similar vascular responses which, however, occur 10 to 20 hours earlier. In preliminary studies, an antihistaminic agent, neo-antergan, was without effect on the reabsorption function, but moderately depressed transudation. It is postulated from this evidence that free histamine is not an important chemical mediator of the vascular changes consequent to UV irradiation. A potent, long acting adrenergic

blocking agent, SKF-688A, administered prior to irradiation had the effect of depressing the reabsorption process more severely, without proportionate altering the transudation pattern.

*Mechanism of excitation of internal secretion of pylorus and adenteric reflex.* ROBERT K. S. LIM AND PHILLIP MOZER.\* Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

Observations on dogs with demyentericized pyloric pouches (mucosa entirely separated from overlying muscle and myenteric plexus; blood supply re-established by apposing omentum or replacing muscle covering) and Heidenhain and/or Pavlov fundus pouches, show that the demyentericized pyloric mucosa responds to local mechanical and chemical (acetylcholine, pilocarpine and histamine) stimulation by liberating gastrin. The pyloric response to mechanical stimulation can be inhibited by 5% cocaine locally, 10 mg/kg. tetraethylammonium intramuscularly, and 0.25% atropine locally, cocaine being the least effective. The response to chemical stimuli is fully inhibited only by atropine, being slightly depressed by tetraethylammonium, and little or not affected by cocaine. The differential behavior of the inhibitors to mechanical and chemical stimulation rules out depression from cellular intoxication, as well as an atropinic effect by tetraethylammonium, and supports the assumption that their action is exerted on the neural sites usually attributed to them. Since the pylorus remains excitable after vagal and myenteric fibers have degenerated following demyentericization, and before regeneration could have occurred, an axon reflex through these fibers can be excluded. Excitation must therefore be mediated by intramucosal neurones. These may consist entirely of secretory efferent neurones which form the terminal relay of an extramucosal chain, or they may include both efferent and afferent neurones. The action of tetraethylammonium in the demyentericized mucosa favors the latter, suggesting that mechanical and chemical excitation of the pyloric glands involves an adenteric (or adeno-enteric) reflex. Chemical stimuli may also excite the glands directly thus shortcircuiting the reflex mechanism.

*Effect of posture on hypertension induced by sympathomimetic amines in man.* A. LITTMAN, R. M. GUNNAR, M. I. GROSSMAN AND R. CASAS. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

In 11 experiments on 6 atropinized human subjects the pressor responses to doses of 0.1 to 0.8 mg. of L-arterenol base were abolished on tilting to the upright position. While upright the subjects felt well, the symptoms associated with the hypertension having disappeared. The hypertension and symptoms returned on tilting back to the horizontal. The drop in blood pressure in the vertical position was proportional to the height of

the pressure level before tilting. Drops in pressure greater than 100 mm. Hg were observed several times. In response to either arterenol alone or atropine alone in normal subjects no such drops in blood pressure occurred with tilting. One- to 2-mg. doses of atropine were given to 7 patients with essential hypertension. No significant drop in pressure occurred with tilting. We interpret this as evidence against the presence of circulating pressor amounts of arterenol, suggested by Goldenberg as a mediator in essential hypertension. We also tested the tilt response after subcutaneous injection of pressor doses of natural and synthetic epinephrine, with and without added arterenol in 21 experiments on 8 normal subjects. This was done in an attempt to reproduce the postural hypotension reported by Smithwick in 4 of 9 patients with hypertension due to pheochromocytoma. No postural hypotensive responses occurred. We interpret this to indicate that hypertension in patients with pheochromocytoma may be due to factors other than circulating epinephrine and arterenol.

*Conditions of ocular damage due to microwave irradiations.* DONALD LOMAX, ALFRED W. RICHARDSON\* AND HAROLD D. GREEN. Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

Previous studies have demonstrated that the application of microwave irradiations may result in irreversible densities in the eye. With the aim of further clarification of conditions contributing to such damage, experimentation has been conducted on 32 albino rabbits *in vivo* in which the eye has been irradiated by 12.25-cm. wavelength microwaves and periodic examination made. The most frequent damage occurred in the posterior portion of the lens with some pathology observable in the lens anterior to this. Typically, impairment was evidenced first as a macroscopic translucent heterogeneous haze of fine particles which became more floccular with time, subsequently forming an opaque mass or masses in the lens, the magnitude varying somewhat with the quantity of irradiation. In the experiments conducted, all damage was observed to occur from one to 14 days following irradiation. It would appear from these studies that relatively precise conditions of energy input may be necessary for opacity formation and that these conditions include energy, distance, and time. In 11 animals exposed to 100 watts power output at a 6-inch distance for 20 minutes, no opacities were observed. Distances of 3.5, 3.75 and 5.0 cm. resulted in 75, 50 and 25% opacity formation respectively with the same power output and time of exposure. Damage may be caused with the pupil of the eye dilated or constricted, if the energy input into the eye is of sufficient magnitude.

*Pyloric sphincter motility and gastric evacuation as affected by vagotomy.* HORTENSE LOUCKES\* AND J.

P. QUIGLEY. Dept. of Physiology, Univ. of Tennessee, Memphis.

The inductographic technic of Brody and Quigley was employed in association with roentgenological observations of a barium meal to study pyloric sphincter activity and the process of gastric emptying. Trained, unanesthetized dogs were extensively studied before and after intrathoracic sectioning of the vagi. The behavior of the pyloric sphincter following vagotomy closely resembled that of the normal sphincter. The sphincter was never in spasm but was either relaxed during rest periods or executing rhythmic movements during periods of activity. Following an 18- to 24-hour fasting period the sphincter was relaxed more of the time than in control animals, but after feeding, the frequency of sphincter contractions was in the normal range. When a bland, fat-free barium meal was fed to a vagotomized dog, it remained in the gastric body and fundus and little mixing occurred for 30 to 120 minutes. The pyloric antrum remained empty, no material passed the incisura angularis and no gastric evacuation occurred. Subsequently, evacuation followed the normal mechanism but was slow and frequently was incomplete, i.e. a gastric residue remained. The delay in onset of gastric emptying, the slow evacuation and the gastric retention resulted from a complete or partial suppression of gastric motility. The sphincter was also somewhat depressed but otherwise behaved normally, and was in no way contributory to the failure of evacuation.

*Effects of temperature on spontaneous and induced electrical activity in cerebral cortex of golden hamster.*

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During hibernation the body temperature of the golden hamster may drop to 2.5°C. Arousal from hibernation is characterized by a gradual autogenous increase in body temperature to 37°C. Under anesthesia the non-hibernating animal can be chilled to body temperatures (5°C.) which would be lethal to mammals which do not hibernate. Investigation of the electrocorticogram of the arousing hibernator reveals no conspicuous activity until the cortex reaches 19° to 21°C. Slow low-voltage activity is the first to appear, to be replaced at higher temperatures by spontaneous burst activity and, at about 29°C., by very fast frequency low-voltage discharges. Local strychninization does not produce convulsive activity until the temperature reaches levels at which spontaneous activity normally appears. Though the cortex appears quiescent early in arousal, peripheral movement can be elicited at temperatures as low as 12°C. by electrical stimulation of motor areas and, under Nembutal anesthesia, peripheral stimulation will evoke a complex cortical response down to 9.1°C. Under Nembutal hypothermia spontaneous activity disappears at 17°C. and reappears at 19°C. During

progressive hypothermia the frequency of induced strychnine spikes gradually diminishes until they disappear at about 15°C. The results are discussed in the light of recent knowledge of the components of the spontaneous electrocorticogram. It is concluded that the bulbar reticular activating system is least resistant to cold; the intralaminar thalamo-cortical non-relay circuits next in resistance; the spino-thalamo-cortical systems and the cortex itself more resistant, and finally (as shown by previous work) peripheral nerve most resistant.

*Albumin-globulin distribution of phytotoxic sera.* DAVID

I. MACHT. Pharmacological Research Lab., Sinai Hospital, Baltimore, Md.

Employing plant-physiological methods for the study of blood for the past 25 years, the author demonstrated the presence of specific phytotoxic substances in one physiological and several pathological human conditions. The usual technique employed is measuring of root-growth of *Lupinus Albus* seedlings in Shive's solutions under precise ecological conditions, with and without admixture of 1% blood serum. The phytotoxic index is the ratio of growth in serum solution divided by growth of controls, and is 70% to 80% for normal sera. The following give toxic indices which are useful for diagnoses and for evaluation of therapy. Menstruation (51%-60%), pernicious anemia (44%-57%), pemphigus (48%-60%) and various psychoses (30%-62%). Recently the author studied the relative phytotoxicity of albumin and globulin fractions in these conditions. Serum globulins were salted out with Na<sub>2</sub>SO<sub>4</sub> and dissolved in Shive's solution. The albumin fractions were freed from Na<sub>2</sub>SO<sub>4</sub> by dialyzing and similarly dissolved in Shive's. Phytopharmacological tests revealed that menotoxin and toxins of P.A. and pemphigus are localized entirely in the serum globulin. The phytotoxins of various psychoses are for the most part confined to the globulin, but may be detected in the serum albumins. A special test for psoriasis blood by using vernalized seedlings was described by the author (*J. Invest. Dermat.* xiii: 1, 1949). Employing this test it was found the phytotoxin of psoriasis is confined to the serum albumin fraction.

*Effects of d-amphetamine on hemi-decorticate, decorticate, and decerebrate cats* (Motion picture). MARTIN B.

MACHT. Dept. of Pharmacology, Cincinnati College of Medicine, Cincinnati, Ohio.

Studies have been made of the effects of intramuscularly administered d-amphetamine sulfate (5 mg/kg. body weight) on the placing, righting, and locomotor responses of one hemi-decorticate, two totally decorticate, and six decerebrate cats. In all preparations the effects of the d-amphetamine became manifest within 30 minutes after administration and disappeared within six to eight hours. In 15 of 17 experiments performed on the hemi-decorticate animals, 5 to 8 months post-

operatively, administration of d-amphetamine resulted in the appearance of previously absent non-visual placing responses in the forelimb and hindlimb contralateral to the ablation. Corrective hopping responses were questionably augmented. Similar results were observed in two totally decorticate preparations studied up to 38 days post-operatively (10 experiments). In addition to the effects on placing responses, however, administration of d-amphetamine to these animals resulted in a definite increase of righting and locomotor activity. The latter effects became less evident as the animals spontaneously recovered locomotion with the passage of time. Administration of d-amphetamine to the six decerebrate animals (38 experiments) resulted in a) righting and locomotor activities earlier in the post-operative period than would be expected in chronic decerebrate cats without amphetamine, and b) corrective placing responses which do not return in chronic decerebrate preparations without amphetamine. The decerebrate animals were studied from 3 to 52 days post-operatively.

*Effect of removal of the liver on blood coagulation.* F. D. MANN, E. S. SHONYO\* AND F. C. MANN. Mayo Foundation, Rochester, Minn.

Recently there have been decided changes in concepts regarding some of the factors concerned in blood coagulation. In a study of the blood coagulation system of dogs following removal of the liver, we have used the following techniques: one-stage and two-stage prothrombin determinations, co-thromboplastin assays, tests for the labile factor and fibrinogen. Hepatectomy was performed by the three-stage method and the animals were maintained in good condition throughout the periods of study, which ranged from 22 to 29 hours. A rapid and progressive decline in all factors studied was observed following hepatectomy. While there were appreciable variations in individual rates of decline, co-thromboplastin consistently decreased more rapidly than prothrombin. Our observations are consistent with the view that normal levels of the factors studied are continuously maintained by the liver.

*Effects of humoral agents on cortical evoked potentials in monosynaptic preparations.* AMEDEO S. MARRAZZI AND ELLEN EVA KING.\* Toxicology Section, Med. Division, Army Chemical Center, Md.

The effects of humoral agents on optic cortical potentials evoked by electrical stimulation of the optic nerve in the cat have been reported by Marrazzi and Hart (*Federation Proc.* 9: 85, 1950), who attribute them to actions on either or both synapses traversed—the geniculate and cortical. The optic cortical synapses have now been studied in isolation by using an intercortical transcallosal pathway of two neurones with a single synapse located in the cortex (Curtis and Bard preparation). Electrical stimulation of the lateral gyrus evokes in the contralateral cortex a potential consisting charac-

teristically of an initial surface positive component, attributable to the presynaptic or ascending callosal fiber from the stimulated cortex, followed by a surface negative component, attributable to the postsynaptic neurone descending from the cortex underlying the recording electrodes. Under favorable circumstances, this constitutes a monosynaptic preparation affording a simultaneous record of impulses delivered to and impulses transmitted by the synapse. The submaximally activated preparation responds to ipsilateral intracarotid injection of minute doses of acetylcholine with synaptic stimulation and to larger doses with synaptic depression, in each case with little or no effect on the presynaptic impulse. Anticholinesterases produce similar effects which can be relieved by atropine. Small doses of adrenaline, in contrast to acetylcholine, produce a primary depression. Thus synapses of the optic cortex appear capable of activation by acetylcholine and inhibition by adrenaline in doses within the range of physiological significance, when they are brought to the synapses by the blood stream. Furthermore, the effect of intracarotid anticholinesterases suggests that a local source of acetylcholine is available in the cortex, presumably at the synapses studied.

*Physiological significance of differences in composition of nucleic acids of nucleus and cytoplasm.* A. MARSHAK. New York Univ. College of Medicine, New York City.

The purine and pyrimidine content of DNA and PNA of isolated nuclei and cytoplasm of calf liver, kidney, thymus and heart have been determined. The composition of DNA is the same in all tissues and contains equal amounts of adenine and thymine and of guanine and cytosine, the latter two being present in amounts 10 to 20% less than the former. The various nuclear pentosenucleic acids (N-PNA's) all have approximately the same guanine-adenine ratios (0.9-1.0), but highly variable and low pyrimidine contents. The cytoplasmic pentosenucleic acids (C-PNA's) as a group also have similar guanine-adenine ratios but in contrast to the N-PNA's the ratios are 1.8-1.9. The proportions of pyrimidines relative to adenine in the C-PNA's are all several times greater than those of the N-PNA's. Pyrimidine ratios of C-PNA's are less variable than those of the N-PNA's. Heart C-PNA has ratios distinctly different from those of other tissues and has no detectable uracil. Previous experiments showed that N-PNA may be the precursor of C-PNA but the reverse is not possible. The present data show that the guanine-adenine ratios of C-PNA may be derived from those of N-PNA by the loss of one  $\mu$  of adenine. The results thus suggest that the free adenine nucleotides of the cytoplasm are produced from N-PNA on its conversion to C-PNA. The varying N-PNA's indicate that cell differentiation involves changes in composition of nuclear constituents. Unusually low cytosine content of thymus N-PNA may be correlated with unusual

behavior of the lymphocyte nucleus toward ionizing radiation.

*Narcotic effects of nitrogen and argon on the central nervous system of frogs.* JEAN M. MARSHALL AND WALLACE O. FENN. Dept. of Physiology, Univ. of Rochester School of Medicine and Dentistry, Rochester, New York.

The narcotic effects of increased pressures of argon and nitrogen on the brain waves and reflex activity of frogs have been investigated. The animals were exposed to pressures up to 81 atmospheres in a chamber containing one-fifth atmosphere of oxygen. Nitrogen at 54 atmospheres caused a complete cessation of brain waves within 15 minutes whereas argon at 41 atmospheres abolished the brain waves in 23 minutes. Below these pressures neither gas was effective. This inhibition was reversible with decompression. Helium at pressures up to 81 atmospheres exerted no observable effects for periods up to 6 hours. Action potentials arising in the tibial nerve after central stimulation of the peroneal nerve served as an index of reflex activity in frogs whose brains had been destroyed. Nitrogen at 17 atmospheres abolished the reflex in about 269 minutes with increasing effectiveness up to a minimum time of 10 minutes at 61 atmospheres. Argon at 10 atmospheres abolished the reflex in 264 minutes with a minimum time of 12 minutes at 61 atmospheres. Below these pressures neither gas was effective. Reflexes returned after decompression. Helium at pressures up to 81 atmospheres was ineffective. No effects were observed on isolated peripheral frog nerves. Thermodynamic activities for threshold narcotic concentrations of nitrogen and argon for inhibition of reflex activity were nearly equal whereas twice as much argon as nitrogen was dissolved in the lipids. Similar results were obtained for inhibition of brain waves.

*Changes in renal function accompanying acclimatization to reduced barometric pressure.* LOUISE H. MARSHALL AND HEINZ SPECHT. Lab. of Physical Biology, Exptl. Biology and Medicine Institute, Natl. Institutes of Health Bethesda, Md.

Serial renal and circulatory measurements have been made at 2-week intervals on 4 dogs exposed intermittently to progressive stages of lowered barometric pressure in the decompression chamber. As expected, the rise in relative viscosity and specific gravity of whole blood paralleled the rise in hematocrit values. Mean arterial blood pressures were slightly elevated. No significant change occurred in NaCN circulation times. The most striking renal change was found in effective renal blood flow, as measured by PAH clearance corrected for hematocrit readings. These flows increased to an average maximum of 80% (+56 to +156%) over control ground level values after 6 weeks intermittent exposure to 20,000 ft. pressure-altitude. One animal showed an initial fall. Glomerular filtration (plasma

creatinine clearance) was elevated consistently (average maximum +35%), and since the effective plasma flow increased slightly or in one case decreased, the filtration fraction rose significantly (+25% average after 6 weeks at 20,000 ft). These results suggest extensive dilatation in the renal vessels, with predominance in the afferent arterioles. Changes in maximum tubular excretory capacity for PAH were slight and not consistent. All blood and renal clearance measurements showed a tendency to return towards ground level values before the low pressure exposures were discontinued, and were within the control ranges four weeks after the last exposure.

*Relative contribution of various environmental climatic stresses to physiological strain experienced by two subjects at work in a hot, dry climate.* F. N. MARZULLI, E. A. MACFADDEN AND I. A. DEARMON, JR. (introduced by DAVID B. DILL). Med. Division, Army Chemical Center, Md.

The relative potency of climatic stresses in producing physiological strain in subjects at work in a hot, dry climate was studied by establishing an arbitrary index of physiological strain derived from changes in rectal temperature, nude weight and pulse and analyzing the contribution of each of the stresses by means of a multiple correlation analysis. The environmental stresses with which we were concerned were ambient temperature, solar radiation, wind, relative humidity and ground temperature. Work experiments were performed in which the normal changes in climate occurring from day to day were utilized in order to learn something of the interaction of the 5 separate environmental stresses in producing physiological strain. Sixteen experiments were performed with one subject and 12 with another at Dugway Proving Ground, Utah, in the summer of 1949, where the ambient temperature ranged from 82.5°F. to 101°F., wind from 1.9 to 5.7 mph, solar radiation from 0.9 to 1.2 gm. cal/cm<sup>2</sup>/minute, relative humidity from 7 to 20% and ground temperature from 94° to 140°F. In these experiments, the prediction of physiological strain could be made quite accurately, with only the data on ambient temperature with little loss from excluding the other factors. Ambient temperature was about 27 times and solar radiation 7 times as effective in predicting physiological strain as ground temperature within the limits of these experiments. Wind velocity and relative humidity were respectively 5 and 4 times as effective as ground temperature.

*Demonstration of antidotal action of polyphosphate in metal poisoning.* ELLIOTT A. MAYNARD, HAROLD C. HODGE AND WILLIAM L. DOWNS. Division of Pharmacology and Toxicology, Dept. of Radiation Biology, School of Medicine and Dentistry, Univ. of Rochester, Rochester, N. Y.

Uranyl nitrate hexahydrate ( $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ) when

fed at a 2% dietary level, has been demonstrated previously in this laboratory to cause kidney injury that is lethal in 21 days to approximately 50% of male albino rats (5 months old). Polyphosphate forms a very stable complex with uranyl ion (Rothstein & Meier, 1950), and calcium polyphosphate (a salt of commercial hexametaphosphate) is in part excreted in the urine after parenteral administration (GOSSELIN *et al.*, 1950). The question arose whether parenterally administered polyphosphate may form a complex with uranium in the body and thus be an effective antidote for uranium poisoning. Four groups of male albino rats (5 months old) were placed on diets containing 2% UNO<sub>3</sub> and were administered hexametaphosphate (as a calcium salt) intraperitoneally as follows: *I*) controls, no polyphosphate; *II*) 2 doses at 4-hour intervals first day; *III*) 2 doses daily, 5 days; *IV*) 1 dose daily, 5 days. Each dose contained the equivalent of 50 mg. P/kg. of body weight. After 21 and 45 days on the diet mortalities were as follows:

	21 days	45 days
<i>I</i> , controls (no polyphosphate)	10/25	13/25
<i>II</i> , 2 polyphosphate injections first day	2/25	13/25
<i>III</i> , 2 days polyphosphate injections, 5 days	0/25	1/25
<i>IV</i> , 1 daily polyphosphate injection, 5 days	2/25	5/25

From these data it is evident that polyphosphate treatment for 1 day delayed death for some rats; repeated treatment for 5 days greatly reduced mortalities after 45 days on the diet. In this preliminary experiment parenterally administered polyphosphate appeared to have marked antidotal properties in uranium poisoned rats.

#### *Can mammary growth inhibit initiation of lactation?*

JOSEPH MEITES. Dept. of Physiology and Pharmacology, Michigan State College, East Lansing.

Excellent mammary growth was induced in intact and castrated male rabbits by injecting 960 IU of estrone and 1 IU of progesterone daily for at least 25 of a 35-day experimental period. From the 25th to the 35th day, each rabbit was injected with 40 IU of prolactin (Squibb) daily, at the end of which time the rabbits were killed and the mammary glands were rated (1-4) for intensity of lactation. Fifteen rabbits received the two steroid hormones for 25 days, and only prolactin during the next 10 days. Their mammary glands were filled with milk and rated 3 to 4. Another 15 rabbits were treated similarly, except that the ovarian hormones were injected during the full 35 days. Only little milk was found (rated 1 or less), indicating that growth can inhibit lactogenic action. Ten rabbits were given the two steroids for 25 days, and the following 10 days 5 received estrone and prolactin and 5 progesterone and prolactin. Full lactation was observed (rated 3-4), showing that neither ovarian hormone interferes with

lactogenic action when given individually. It is concluded that the mammary-growth action of estrone and progesterone, when given together but not individually, can counteract the initiation of lactation by prolactin. The two ovarian hormones may act in a similar manner during pregnancy to inhibit lactation, although the low prolactin secretion by the pituitary during gestation must also be considered.

#### *Skin, rectal and intravascular temperature adjustments in exercise.* HENRY C. MELLETTE (introduced by H. C. BAZETT). Dept. of Physiology, Univ. of Pennsylvania School of Medicine, Philadelphia.

In a study of temperature changes occurring in the vascular tree and in deep and superficial tissues of the human body thermocouples were placed in the subclavian artery, inferior vena cava, rectum and dermis of the thigh in 3 healthy men aged 24 to 26. The experiments, carried out on the nude subjects, untrained in bicycle ergometry, consisted of one-half hour's rest on a cot, 60 minute's work at rates of 384 to 801 kg.m/minute on the ergometer and 1 hour's recovery on the cot in a temperature-controlled room at an effective temperature of 21°C. (69.5°F.). The temperature in the inferior vena cava (5-8 cm. below the diaphragm) was found to rise from an average resting value of 37.4° to 38.9°C. at the end of 60 minutes of work in 4 experiments. Corresponding values for the artery and rectum were 37.3° to 38.5°C. and 37.0° to 38.3°C. respectively. Average rates of temperature change in the first 15 minutes of work were 0.08°C/minute in the vein, 0.07°C/minute in the artery and 0.03°C/minute in the rectum. In moderate and moderately heavy work (up to 678 kg.m/min.) a leveling off of central venous and central arterial temperatures took place within 19 minutes while in the rectum this occurred 35 minutes after the beginning of work. In very heavy work (801 kg.m/min.) no such plateauing of these temperatures was observed. Arterial and venous temperatures commonly returned to pre-work levels during the first half of the recovery period but often the rectal had failed to do so at the end of 1 hour. The general pattern of dermal temperature changes was an abrupt rise during the first quarter of the exercise period followed by a slow decline approaching resting values at the end of 60 minutes. During the 1-hour recovery an abrupt rise was again followed by a cooling but in the phase was sufficiently slow that resting values were not commonly attained.

#### *Perfused frog heart in assay of cardiotropic substances.*

ARTHUR E. MEYER AND JACK GREENBERG.\* Fellows Pharmaceutical Lab., New York City.

Hearts of *Rana pipiens* were perfused from the vena cava at 2 cm. water pressure against a resistance of 6 to 7 cm. pressure. Cytochrome C added to the perfusion fluid caused a gradual decrease in stroke volume at unaltered heart rate. Adenosine-5-monophosphate reduced

the heart rate markedly with increased stroke volume keeping the total volume unchanged. Epinephrine increased the output mainly by faster rate. This rate increase was abolished by adenosine monophosphate. Adenosine triphosphate gave variable stimulation. Veratramine, veratrosine, jervine and pseudojervine lowered the heart rate and counteracted the acceleration by epinephrine. Longer action or high dosage impaired the stroke output. Rubijervine, protoveratrine, isorubijervine, germinine and germerine did not affect the heart rate but progressively reduced the output. The action of the various agents in reversible as long as there is no decline in stroke output, thereafter washing out accelerates this decline, which process may be temporarily delayed by re-application of medication. The adenosine compounds on long application cause arrhythmias with extra-ventricular beats, the veratrum alkaloids more often produce heart block. A mechanism of mutual compensation between rate and stroke output to maintain constant minute output is abolished by the decelerator alkaloids but not by adenosine monophosphate.

*Effects of localized cortical destruction upon auditory discriminative conditioning in the cat.* DONALD R. MEYER and CLINTON N. WOOLSEY. Dept. of Physiology, Med. School, Univ. of Wisconsin, Madison.

Cats were trained to discriminate small increments in the frequency of auditory stimuli. The background noise level of the experimental room was approximately 50 db S.P.L. Each trial of the 20 in a session began with presentation of a varying number of 1000 cycle tones at 65 db S.P.L., each 2 seconds in duration and spaced one second apart. The series was followed by a 1100 cycle tone reinforced with shock and buzzer. The animals learned to avoid shock by running in a rotating cage. Generalization training began after a criterion of 90% responses was reached, and critical tests were given at increments of 20, 40, 60, 80 and 100 cycles. Cats were retrained by the same procedure after production of a variety of symmetrical cortical ablations, the most extensive of which removed all neocortex posterior to somatic area I. Attempts were made to produce discrete destruction of known auditory fields in combination and isolation. Extirpations of auditory area I; somatic area II; somatic area II and Ep; auditory area I, somatic area II, and Ep; auditory area II, somatic area II, and Ep; and auditory areas I and II, Ep, the cerebellar tuber vermis, suprasylvian gyrus, and temporal region are without effect upon frequency discrimination. A lesion that combined destruction of somatic area II, Ep, and the middle region of auditory areas I and II failed to produce a decrement, but any lesion that included complete removal of all these areas resulted in a lasting impairment. Animals that could not discriminate frequency could acquire a simple conditioned response and discriminate small changes in intensity.

*Fluorescein studies of circulation time in monkeys with lucite calvarium.* DAVID MINARD. Naval Med. Research Institute, Bethesda, Md.

Monkeys fitted with lucite skull plates received injections of sodium fluorescein in the leg veins. Appearance time and passage of the dye in the pial vessels were observed while the brain surface was illuminated with filtered ultraviolet light. No significant staining of the brain surface occurred. Hence the determinations could be repeated at short intervals. Automatic injection of dye, and objective recording by cinéphotographic or photoelectric means, eliminate errors in timing inherent in the usual methods using manual injection and visual observation. With cinéphotographic recording, it was possible to determine cerebral artery to cerebral vein time (cerebral circulation time). With photoelectric recording the time from initial fluorescence to maximum fluorescence was measured instead. Uniformity of injection rate is necessary to obtain valid and consistent values since rapid injection tends to shorten circulation time. With slow injection rates, on the other hand, the initial appearance of fluorescence may be overlooked by the observer. Hypoxia and histamine prolonged the appearance time of fluorescein at the brain surface and slowed the rate of spread through the brain vessels. Epinephrine in larger dosage prolonged circulation time and in smaller dosage shortened it slightly. These effects are largely cardiovascular in origin. Local action of these agents on the pial vessels is probably of lesser importance since 10% carbon dioxide which produces marked local dilatation has little consistent effect on circulation time.

*Treatment and prophylaxis of experimental renal hypertension with crude hog renin.* J. B. MOORE,\* BESS OSGOOD,\* E. W. HAWTHORNE\* AND G. E. WAKERLIN. Dept. of Physiology, Univ. of Illinois College of Medicine, Chicago.

In previous studies by our research group on the effect of hog renin in the treatment of chronic renal hypertensive dogs and in the prevention of experimental renal hypertension following bilateral renal artery constriction, good and variable therapeutic and prophylactic results were obtained. Frequently however, the antihypertensive effects were questionably or not correlated with the antirenin titres. Cumulative evidence suggested that this lack of correlation was due to variable contamination of the renin with renal medullary protein which appeared to have an interfering action on the renin-antirenin antihypertensive effect. In experiments reported here, crude hog renin from which all renal medulla had been excluded was used in the therapy and prophylaxis of experimental renal hypertension. Eight chronic hypertensive dogs received daily intramuscular injections of crude hog renin, 2.5 DU (Goldblatt)/kg. (1 DU/mg. N) for periods ranging from 5 to 8 months. Four of the 8 showed significant reductions in blood pressure, 3 of the 4 to normotension.



Four prophylactic dogs received the extract for 4 months previous to the second renal artery constriction. Three of these dogs showed complete protection against the development of hypertension, while one showed no protection. Good correlation was observed between the antirenin titres and the therapeutic and prophylactic results obtained. These findings, together with others previously and presently reported by our research group, are strong support for antirenin as the mechanism of the antihypertensive effects of hog renin. Our results also constitute important positive evidence for the renin-hypertensin concept of the pathogenesis of experimental renal hypertension.

*Study of relationship of arterial oxygen tension to alveolar oxygen pressure in man, utilizing a polarometric method for whole blood.* EDWARD H. MORGAN AND GABRIEL G. NAHAS (introduced by EARL H. WOOD). Mayo Foundation, Univ. of Minnesota, and Mayo Clinic, Rochester, Minn.

In 10 normal subjects breathing gas mixtures containing 40.5, 50.6, 60.8, 69.5 and 99.5% oxygen, 66 simultaneous measurements of alveolar oxygen pressure and arterial oxygen tension were made. Alveolar  $PO_2$  was measured with a Beckman oxygen analyzer in gas obtained with a continuous end expiratory sampler. Arterial  $PO_2$  was measured polarometrically with a rotating platinum electrode. In supine subjects breathing normally, the average values for arterial  $PO_2$  were consistently lower than alveolar oxygen pressures, the difference ranging from -7 mm. Hg at an average alveolar  $PO_2$  of 236 mm. Hg to -57 mm. Hg at an average alveolar  $PO_2$  of 637 mm. Hg (average barometric pressure = 733 mm. Hg). Upon increasing depth of breathing, 6 supine subjects breathing 99.5% oxygen exhibited elevation of arterial  $PO_2$  and decrease in the difference of arterial  $PO_2$  from alveolar  $PO_2$ . A likely reason for the observed reduction in the oxygen tension deficit of arterial blood was that deep breathing produced an increase in aeration of alveoli perfused but poorly ventilated during normal breathing, thus reducing the fraction of blood by-passing well-aerated alveoli. Data obtained from 6 subjects, in whom minute volume of ventilation was measured during breathing of the gas mixtures, revealed slight but statistically significant increase in minute volume with respect to increase in alveolar  $PO_2$  amounting to approximately 2 ml/mm. Hg increase in alveolar  $PO_2$ . Theoretical calculations indicate that the average arterial oxygen tension deficits found in the present study could be explained by the assumption that in the normal supine subjects approximately 2% of the blood flow through the heart by-passed aerated alveoli.

*Production of hypertension following choline deficiency in weanling rats.* CAMPBELL MOSES, GRACE M. LONGABAUGH AND ROBERT S. GEORGE. Addison H.

Gibson Lab., Univ of Pittsburgh School of Medicine, Pittsburgh, Pa.

Recently, Hartroft and Best (*Brit. M. J.* 1: 423, 1949; *Federation Proc.* 8: 610, 1949) have demonstrated that weanling rats surviving the renal damage resulting from a brief period of choline deficiency (*J. Biol. Chem.* 131: 567, 1939) have a high incidence of hypertension. Sobin and Landis (*Am. J. Physiol.* 148: 557, 1947) have not found any evidence of hypertension in rats maintained on a choline deficient diet over long periods of time. This study was undertaken to determine the effect upon blood pressure at maturity of varying degrees of choline deficiency for a 5-day period after weaning and to observe the effect of a similar choline deficiency on more mature rats. Three groups of weanling rats of the Sprague-Dawley strain (average weight 43 gm.) were fed 1) a choline free diet, 2) the same diet plus 400 mg. choline chloride/kg. diet, and 3) with 2 gm. of choline chloride/kg. added to the diet for a period of 5 days. At the end of this time representative animals from each group were sacrificed and the remaining animals restored to a normal diet (Rockland Rat Ration) for 6 months until sacrifice. During this period indirect blood pressure determinations were made by the method of Kersten, *et al.* (*J. Lab. & Clin. Med.* 32: 1090, 1947). Under sodium pentobarbital (Nembutal) anesthesia at the time of sacrifice direct blood pressure readings were obtained by cannulation of the abdominal aorta. Three groups of older rats of the same strain (average weight 140 gm.) were given similar diets for 10 days followed by sacrifice of representative animals and the administration of a normal diet for 6 months. Of 28 weanling rats surviving a 5-day choline deficient diet and maintained on a normal diet for 6 months thereafter 20 animals or 71 per cent had arterial blood pressure levels over 150 mg. at the time of sacrifice. Similar groups of animals receiving an 0.4 mg.% or 2.0 mg.% choline diet and older rats (140 gm.) surviving 10 days of similar diets did not have demonstrable hypertension at sacrifice 6 months later. (This study was made possible by a grant from the American Foundation for the Study of High Blood Pressure. Support was also received from the Sarah Mellon Scaife Foundation.)

*Studies on maximal breathing capacity measurements.*

HURLEY L. MOTLEY AND JOSEPH F. TOMASHEFSKI. Cardio-Respiratory Lab., Barton Memorial Division, Jefferson Hospital, and Dept. of Medicine, Jefferson Med. College, Philadelphia, Pa.

Maximal breathing capacity (MBC) measurements provide valuable information concerning pulmonary function, however, no standardization of the test technique exists. A modified Benedict-Roth spirometer (no inside valves or cannister) was used designed for minimal breathing resistance by employing a bell 22.9 cm. diameter (capacity 13.5 liters) and large stiff tubing (inside diameter 3.3 cm.) to connect the high velocity

valve (Rudolf or Bishinger-Koehler). Repeated tests were performed to determine the highest MBC value (taken standing for 12 seconds per run and computed on minute basis) with varying rates and depth of breathing (recording by two writing pens on a kymograph) and adequate rest intervals. Check MBC values (less than 5% variation) were obtained in all types of untrained but cooperative individuals and over a wide range of measurements (12 to 248 l/minute). Most subjects were unable to maintain a constant state of maximal deep fast breathing for more than 15 seconds, for longer time intervals predisposed to bronchospasm. Electrical measurements of MBC using a 3-inch diameter monel screen (400 mesh) were not significantly higher. The predicted  $MBC = 97 - (0.5 \times \text{age in yr.}) \times B.S.A. M^2$ , with an observed S.D. of  $\pm 11.5\%$  and S.E. of  $\pm 3.5$  l/minute in young men (20-30 yrs.). The correlation noted between MBC and pulmonary emphysema (based on quantitative measurements of residual air) in 432 miners was as follows: if the MBC was less than 40 l/minute the degree of emphysema was significant, insignificant if the MBC was over 120 l. and otherwise indeterminate.

*Adrenalectomy on oxygen consumption of frogs.* MARY C. MURRAY\* AND CLIFFORD A. ANGERER. Dept. of Physiology, Ohio State Univ., Columbus.

Unfed, male *Rana pipiens*, (winter and spring stock) weighing between 20 and 30 gm. and showing testicular fat at autopsy, were divided into 3 groups: adrenalectomized, unoperated (control) and renal damaged (control) frogs. The oxygen consumption was determined by means of constant pressure respirometers at 30°C. The determinations were corrected to constant pressure and were expressed as ml. oxygen consumed/gm./hour. The mean value for the oxygen consumption for all unoperated winter and spring frogs is  $0.175 \pm 0.003$  ml/gm./hour, or  $0.183 \pm 0.006$  and  $0.165 \pm 0.006$  for these respective groups. Renal damaged frogs gave a mean value of  $0.175 \pm 0.006$  ml/gm./hour on the 5th postoperative day. Adrenalectomized frogs showed a progressive decrement in mean oxygen consumption values along a sigmoid curve. These values are statistically significant after the 5th postoperative day. On the 5th day the mean value for all frogs was  $0.095 \pm 0.007$  ml/gm./hour. This is a decrease of 45.8% ( $P < 0.01$ ) and 52% when compared respectively with renal damaged and unoperated frogs.

*Rapid determination of hemoglobin and oxyhemoglobin in whole hemolyzed blood, using a lucite cuvette adapted to Beckman spectrophotometer* (Demonstration). GABRIEL G. NAHAS (introduced by EARL H. WOOD). Mayo Foundation, Univ. of Minnesota, and Mayo Clinic Rochester, Minn.

A lucite cuvette, allowing for anaerobic analysis, was built to fit the Beckman D. U. spectrophotometer. It consists of 2 lucite plates, a metal gasket 0.01 cm. thick

and supporting plates of nickel-plated stainless steel. The metal gasket and plate contain 4 apertures (so that when they are clamped in place 4 chambers are formed between the lucite plates which are 0.01 cm. in thickness and conform in shape and area to the standard Beckman cuvette holder). Two L-shaped holes are drilled through one of the lucite sheets into each side of the chambers. Filling of the chambers with blood is performed with a Luerlok syringe and a 20-gage rubber-tipped needle, the dead space of which is filled with a few drops of an anticoagulant solution and mercury. Hemolysis of the blood is produced anaerobically by adding 1/100th of the sampler volume of a saturated solution of saponin (Merck). A volume of blood equal to three times the volume of the chamber (20 mm.) is flushed through it in order to avoid any exchange of air between blood and the air filling the cuvette chamber. Analysis of oxygen saturation depends on the differences in spectral absorption of  $HbO_2$  and Hb. at 605 and 505 mm., the isobestic point of oxygen and reduced hemoglobin. The standard deviation between this photometric determination and the Van Slyke determination of oxygen capacity and percentage oxygen saturation of 37 blood samples (ranging from 13.3 to 25.4 gm/100 cc. of hemoglobin) was 0.5 gm. Hb/100 cc. and 1.9% saturation, respectively.

*Oxygen dissociation curve in arterial blood of man breathing high oxygen mixtures using polarographic and photometric methods for whole blood.* GABRIEL G. NAHAS,\* EDWARD H. MORGAN\* AND EARL H. WOOD. Mayo Foundation, Univ. of Minnesota, and Mayo Clinic, Rochester, Minn.

Simultaneous measurements of arterial oxygen saturation and arterial oxygen tension were carried out in 10 normal subjects during inhalation of air, 40, 50, 60, 70 and 100% oxygen. A cuvette oximeter attached to an indwelling arterial needle (WOOD, NICHOLSON. *Am. J. Physiol.* 163: 762, 1950) and a rotating platinum electrode (*Federation Proc.* 9: 91, 1950) were used. The standard deviation of single determinations of oxygen saturation of fully saturated blood samples from the mean value obtained for individual subjects was 0.13% saturation. Sixty determinations of oxygen saturation were performed at arterial oxygen tensions ranging from 197 to 644 mm. Hg. A significant curvilinear increase in oxygen saturation totaling  $0.4 \pm 0.1\%$  was found to occur as the average arterial oxygen tension was increased from 230 to 400 mm. Hg. No further increase in oxygen saturation was demonstrated at arterial tensions above 400 mm. Hg. The average arterial oxygen tension of subjects breathing 70% oxygen was  $406 \pm 9$  mm. Hg. Average increase in arterial saturation in 6 subjects produced by the initial change from breathing 50% to 100% oxygen averaged  $0.3 \pm 0.1\%$ . The decrease upon the change from breathing 100% to 50% oxygen after a period of 70 (30-90) minutes breathing 100% oxygen averaged  $0.7 \pm 0.1\%$  satura-

tion. Such ready reversibility would not be probable if the changes detected were due to conversions of inactive heme pigments in the blood. These findings were interpreted as indicating that *in vivo* a blood oxygen tension of at least 400 mm. Hg is required to effect practically full saturation of hemoglobin in arterial blood (temperature 37° C., CO<sub>2</sub> tension 40 mm. Hg). This can be achieved in most normal subjects by inhalation of 70% oxygen (average barometric pressure: 732 mm. Hg).

*In vivo inhibition of barium chloride stimulated intestinal motility by atropine.* H. NECHELES, WM. SCRUGGS, S. KRAFT AND WM. H. OLSON. Dept. of Gastro-Intestinal Research, Med. Institute of Michael Reese Hospital, Chicago, Ill.

It has been accepted that barium chloride exerts a direct musculotropic action on the intestinal tract, and that atropine has little if any effect on this activity. Acute experiments on dogs under sodium pentobarbital (Nembutal) anesthesia have shown that this is not true. Single balloons were placed in the esophagus and stomach, and double balloons were placed in the jejunum and colon. Barium chloride in saline solution was given i.v. at a constant rate of 0.5 mg/minute to 1.0 mg/minute. When a constant degree of motility was present, i.v. atropine produced a marked inhibition of motility. Instillation of atropine topically in regions of the double balloons had no effect in most experiments, but in a few instances it produced some inhibition. These results were different from those obtained on the isolated intestinal strip of the guinea pig, where atropine had no effect on barium chloride stimulated motility. Studies of effects of antispasmodic drugs on the isolated intestinal strip alone may not give the true pharmacological effect of these drugs, and the current opinion on a pure musculotropic effect of barium may have to be revised.

*Function of auditory cortex: localization of sound in space.*

WILLIAM D. NEFF, G. PETER ARNOTT\* AND JOSEPH D. FISHER.\* Lab. of Physiological Psychology, Univ. of Chicago, Chicago, Ill.

Changes in behavior produced by ablation of auditory areas I and II have been studied by testing experimental animals (cats) in a discrimination situation requiring the localization of sound in space. Unilateral ablation of auditory areas I and II did not affect the ability of animals to respond appropriately. Bilateral ablation resulted in severe deficit in the learned habit. Normal animals were able to make correct discriminations when the angle between successive positions of the sound stimulus was as small as 5°. Operated animals were able to reach the same criterion of performance only for angles of 40° or greater. A more striking difference, however, in the behavior of the operated animals was their inability to maintain a high percentage of correct responses over a period of time. Normal ani-

mals reached a level of 90 to 100% correct discriminations (10 trials per day) and maintained this level indefinitely. Operated animals reached the 100% level under optimal conditions but failed to maintain this level on successive days; typically, they fluctuated between chance and 100%. It is concluded that auditory areas I and II are necessary in order that auditory signals will be utilized most effectively in the control of learned motor behavior. In a simple learning situation such as avoidance conditioning to auditory stimuli the deficit produced by ablation of areas I and II is difficult to detect; in a more complex, less restricted situation such as used in the present experiment, the change in discriminatory ability is striking. (This work was supported by the Office of Naval Research, Contract N6 ori-20, T. O. XXIV, ONR Project NR 140 608.)

*Estimation of cardiac output and blood volume by continuous recording of Evans blue time-concentration curves in man, employing an oximeter.* JOHN W. NICHOLSON, III\* AND EARL H. WOOD. Mayo Clinic and Mayo Foundation, Univ. of Minnesota, Rochester.

Addition of T-1824 increased red light (640 mμ) absorption and very slightly decreased infra-red (800 mμ) absorption of blood. A single-scale cuvette oximeter attached to an indwelling arterial needle was thus used to obtain continuous photographic records of dye concentration in whole blood following i.v. injection of 40 mg. T-1824 in 10 normal subjects and 27 patients. Oximetric quantitation of T-1824 in blood requires constancy of blood oxygen saturation; all subjects breathed 99.6% oxygen during the procedure. Known blood-dye dilutions covering the 5 to 50 mg./l. range embraced by most dye curves were made from arterial bloods drawn just before dye injection in all subjects. Standard deviation of individual calibrations of dye in blood from an average curve of 15 subjects was 6%. Variations in hemoglobin concentration between individuals gave no systematic variations in the dye calibration curve. Cardiac output measurements made by extrapolation and planimetry on the photographic record averaged  $124 \pm 1.4\%$  (Figures following  $\pm$  signs are standard errors of means) of values derived by replotting curves semilogarithmically (method of Hamilton *et al.*). Average normal cardiac index of  $3.17 \pm 0.2$  l/minute/m.<sup>2</sup> by this method closely approximates direct Fick results reported by others. Eight individuals having both Fick and cuvette-dye determinations showed cardiac index differences ranging from +23 to -17% of Fick value without systematic difference. Successive blood volume determinations in 12 normal subjects 90 and 360 seconds after dye injection showed mean values of  $65.4 \pm 2.4$  and  $72 \pm 3.0$  ml/kg. respectively. The standard deviation of the errors of 15 *in vitro* blood volume determinations was 5%. Ear oximeter dye curves recorded in all subjects

and quantitated by calibration against one direct determination of blood dye concentration at 5 minutes yielded an average normal cardiac index of  $3.05 \pm 0.2$ . Records from cardiac patients show diagnostic possibilities.

*Effects of iodoacetate on retina and nerve.* WERNER K. NOELL, USAF School of Aviation Medicine, Randolph Field, Texas.

To correlate the high glycolytic rate of the retina *in vitro* with the maintenance of retinal functions the effect of sodium iodoacetate (IAA) on retinal potentials in response to illumination was measured in various vertebrates. Poison was administered i.v. A single dose of 15 to 25 mg/kg. body weight abolished retinal excitability in rabbit and cat within 2 to 4 minutes as measured by the optic nerve response. In frog ( $21^{\circ}\text{C}.$ ) IAA was ineffective during aerobic conditions, but the survival time of the electroretinogram after decapitation was reduced to less than 10% of the control value. In pigeon and turtle relatively slow effects of IAA were observed under aerobic conditions. There were no measurable effects of IAA on the saphenous nerve (rabbit, urethane narcosis) *in situ* during air breathing; however, its survival time during anoxia induced by nitrogen breathing 20 minutes after the administration of 50 to 100 mg. IAA/kg. body weight was reduced as indicated by table.

ANOXIC SURVIVAL		'A' FIBERS		'C' FIBERS	
TIME (in minutes)		> 40 m/s	< 15 m/s	1.5 - 0.8 m/s	
Controls	n = 5	37	16	62	
After IAA	n = 5	14	5	8	

The temperature of the nerve and surrounding tissue was kept close to  $37^{\circ}\text{C}.$  during anoxia.

*Use of parenteral fluids in hemoglobinemic nephrosis.*

WM. H. OLSON AND H. NECHELES. Dept. of Gastro-Intestinal Research, Med. Research Institute of Michael Reese Hospital, Chicago, Ill.

Intravascular hemolysis was produced in anesthetized dogs with high frequency sonic vibrations. Hemoglobinemia alone did not result in fatal renal insufficiency, even with plasma hemoglobin values as high as 4500 mg. %. Combining hemolysis with a moderate reduction in blood pressure and hemoconcentration by the use of 50% glucose intraperitoneally, resulted in fatal renal insufficiency only when complete anuria was present for 24 hours or longer. Animals recovered completely when spontaneous excretion of urine occurred immediately after hemolysis, or when urine was secreted within 24 hours either spontaneously or following intravenous fluids. When parenteral fluids did not stimulate kidney excretion, pulmonary edema developed readily, causing death within 1 to 3 days. Development of jaundice resulted in death from 3 to 5 days. Animals with complete anuria and no other

complication could survive for 6 to 11 days, their kidneys showing lower nephron nephrosis.

*Influence of an interatrial septal defect on the hemodynamics of mitral stenosis.* D. F. OPDYKE AND G. A. BRECHER. Dept. of Physiology, Western Reserve Univ. Med. School, Cleveland, Ohio.

Mitral stenosis was produced acutely in dogs a) without an interatrial septal defect and b) in the same dog after the production of a defect. Without the defect mitral stenosis resulted in a decrease of systolic and diastolic aortic pressure, marked increase of left atrial pressure and little change of right atrial pressure. The aortic pressure pulse showed a characteristic change of contour. The upstroke became steeper and the pressure declined rapidly during the last half of systole, resulting in a peaked curve. The maximum increase of left atrial pressure occurred at the V point. Systolic left ventricular regurgitation occurred in most experiments. In the presence of an interatrial septal defect mitral stenosis resulted in the same changes of contour and pressure of the aortic curve. Left atrial pressure increased markedly, in some instances to a greater extent than before the defect, although the degree of stenosis was the same. Right atrial pressure increased only slightly. It is concluded that in the acute experiment the superimposition of an interatrial septal defect on mitral stenosis is without detectable dynamic effect.

*A one-inch lucite cranial window and vitallium holder for installation in monkeys.* ELLIOTT F. OSSERMAN, DAVID MINARD AND E. H. REYMANN. Naval Med. Research Institute, Bethesda, Md., and Austenal Lab., Inc., New York City.

A one-inch lucite cranial window with a vitallium holder has been devised for the visualization of the cerebral blood vessels of Rhesus monkeys. The frame consists of a cast vitallium ring with internal threads for the insertion of a threaded lucite window. The vitallium ring is contoured to fit the transverse curvature of the skull, and the flanges used for screwing the ring to the skull are bent to conform to the A-P curvature. This window can be installed in a single operation, and a new instrument for cutting the one-inch hole in the skull has also been developed. The cutting instrument is mounted on a duraluminum ring, and three steel points extending into this ring provide rigid bony fixation of the skull with respect to the cutting instrument. The use of this instrument has greatly facilitated the operative procedure. Using a preliminary cutting instrument, the first two windows were installed and the dura left intact at the original operation. The dura of both of these preparations was subsequently removed after 2 weeks had elapsed to permit healing of the bone edges around the frames. Use of the new instrument permits dural resection at the original operation. The removable window makes possible the debridement of exudate or regenerated tissue. Up to this time the

windows have all been placed over the parietal areas, but it is believed that other areas will also be accessible.

*Does the presence of a person act on cardiac rate of the dog as unconditional stimulus?* OLGA OWENS AND W. HORSLEY GANTT, Pavlovian Lab. Johns Hopkins Univ., Baltimore, Md.

Not only food and other physical stimuli act on the animal, but the presence of a person has been shown to have an effect. With the neurotic dog, Nick, Gantt noted that a person had double and opposite effects: 1) evoking neurotic (CR), and 2) any person petting, i. e. rubbing behind the ears, dissipated the neurotic behavior, (UR?). Cardiac rate was shown by Gantt and Hoffmann to be a most delicate measure of both conditional and unconditional responses. We used this measure in our experiments. There was a definite pattern for each dog, changing little after several weeks' repetitions. Cardiac rate falls to the light signal, rises on entering the room, shows a secondary rise on approaching the dog and a deep drop on petting him. The pattern was equivalent in several dogs tested, and constant for 80 repetitions in a month, except for some lowering of rate. There was evidence of a conditioned deceleration to the light used alone. Constancy of pattern and ease of conditioning indicate that the presence of a person acts as an unconditional stimulus on the cardiac rate of dogs. This is the first of a series of experiments to determine the effect of a person (experimenter) on the experimental animal.

*Nature of cysteine induced radioresistance: sulphydryl levels and distribution of cysteine sulfur.* H. M. PATT, R. L. STRAUBE,\* M. E. BLACKFORD\* AND D. E. SMITH. Argonne Natl. Lab., Chicago, Ill.

Cysteine may increase radioresistance by: 1) decreasing free radical production, 2) competing directly for oxidizing free radicals, 3) shifting the redox potential of critical cell elements, thus protecting them against oxidation, and 4) altering metabolic pathways. Assuming selective action on sulphydryl groups by products of irradiated water, less than 1% of nonprotein -SH (as cysteine) could theoretically be oxidized when a 200-gm. rat is exposed to 800 r. X-irradiation had no immediate observable effect on -SH levels in control and cysteine rats, although cysteine per se (950 mg/kg. i.v.) increased the oxidizable -SH content of TCA filtrates of serum, kidney, thymus, intestine and liver. Rather identical retention of  $S^{35}$  was noted in blood of rats receiving labeled cystine, cysteine, or cysteine followed by X-irradiation, (60 mg. cystine or cysteine,  $5 \times 10^4$  counts/min., i.v.). Uptake of  $S^{35}$  by liver and intestine was more rapid in cysteine injected rats. X-radiation appeared to decrease rate of uptake and retention. The initial concentration of  $S^{35}$  in bone marrow was similar in cystine and cysteine animals but fell sharply during the first hour in the latter. Marrow uptake was diminished by irradiation. No clear re-

lationship between  $S^{35}$  concentration in these tissues and the time course of cysteine protection was evident. Our data do not support the concept of direct or indirect competition by cysteine for oxidizing radicals. If such a mechanism is operating, oxidation must be localized in specific sites where -SH or other essential substances are few and their inactivation critical.

*Physiological factors governing hippuric acid synthesis in man.* HAROLD PERSKY, STANFORD R. GAMM AND ROY R. GRINKER (introduced by SAMUEL SOSKINS). Michael Reese Hospital, Chicago, Ill.

Hippuric acid excretion is markedly altered in certain mental diseases. In order to elucidate the relationship between these psychiatric disorders and a clinical test of liver function, the effect of a variety of factors on this synthesis and excretion were measured. The emotional responses to stress of 'free' anxiety and catatonia raised and lowered the excretion respectively. Removal of the frontal lobe areas 9 and 10 of the cortex alters the excretion in the same direction as it affects 'free' anxiety. Vagal section significantly raises excretion of hippuric acid in individuals with peptic ulcers. The CNS drugs: mescaline and sodium pentothal, lower and raise hippuric acid excretion respectively. The autonomic drugs: acetylcholine, prostigmine, adrenaline and dibenamine, did not affect the excretion, but nor-adrenaline, ephedrine and atropine lowered it significantly. Altered endogenous excretion, bladder retention, kidney function, motor activity or hepatic blood flow are not responsible for these findings. These effects are due to alterations in the rate of synthesis of hippuric acid, particularly the rate of glycine mobilization. The mobilization of this amino acid, like the others, is dependent on various hormones: cortisone and insulin, for example. It is postulated that a cortical-thalamic-hypothalamic regulatory mechanism is responsible for the altered synthesis, probably via the pituitary gland, and that this regulation constitutes an attempt to maintain homeostasis with respect to amino acid metabolism.

*Observations on aortic pulse contour of unanesthetized dogs.* R. W. PICKERING AND R. G. ELLISON (introduced by W. F. HAMILTON). Depts. of Physiology and Thoracic Surgery, Med. College of Georgia, Augusta.

The necessity of obtaining central aortic pressures in dogs repeatedly over long periods of time led to the production of a cannula for this purpose. The cannula consists of a 12 mm. wide silver band which completely encircles the aorta. Silver-soldered to this band is a telescoping silver tube whose distal end is fixed immediately under the skin. An 18-gauge spinal needle is passed through this tube into the aorta and pulse contours are recorded by means of an optical manometer. Preliminary observations were made in animals with neurogenic hypertension produced by removal

of the carotid sinuses, section of the cardiac vagal fibers in the chest and division of the right vagus in the neck. It was found that after extensive dissection of the thoracic vagus, hypertensive dogs were produced which had rapid heart rates, large cardiac outputs and normal resistances. However, if a limited dissection was carried out, hypertensive animals were obtained with normal heart rates, normal cardiac outputs but high resistance values. The latter observations correlate with those seen in clinical and experimental renal hypertension. The heart rates were not slowed by hypertension from the administration of 5.0 gamma/kg. of epinephrine but did accelerate after 1.0 mg. of atropine was administered. These data might indicate that the pressure receptor fibers have been destroyed but that some of the vagal pacemaker fibers were intact.

*Urinary excretion of gonadotropins, 17 ketosteroids and estrogens and estrogen clearance test in patients with chronic hepatic disease.* I. J. PINCUS, A. E. RAKOFF, E. COHN AND H. TUMEN. Jefferson Med. College and Graduate Hospital, Philadelphia, Pa.

The 17 ketosteroid excretion was consistently low in the group of patients studied. This may be a manifestation of malnutrition or any chronic disease as well as reflecting specific changes due to disturbed liver function. The gonadotropin excretion was diminished in some, normal in others. The studies of estrogen excretion were of particular interest. Twenty-two tests on 16 males revealed 7 abnormally high values. Only 2 of these latter patients showed gynecomastia, palmar erythema or spider telangiectasia, whereas 5 patients with normal or low levels showed one or more of these so-called 'estrogen effects.' Of 7 tests on 6 females in the reproductive age, one was above normal in a patient with 'hyperestrogen effects' while 2 other patients with similar manifestations had normal values. Eight tests were performed on 3 females in the post-menopausal period. Of 4 tests performed on the one patient with 'hyperestrogen effects,' one was abnormally elevated. Three tests on the 2 patients with no such manifestations were also above normal. Five of 12 estrogen clearance tests performed on 8 of these patients were abnormal. There was no correlation between the urinary estrogen level and the results of this test. These results suggest that the urinary estrogen and estrogen clearance values are only inconstantly abnormal in patients with hepatic disease, and that the so-called 'estrogen effects' commonly occur in association with normal values. These manifestations may be produced by relative changes in a complex group of factors rather than a simple disturbance of one of these factors.

*Absorption, distribution and excretion of tritium in men and animals.* ERNEST A. PINSON AND ERNEST C. ANDERSON. Los Alamos Scientific Lab. of the Univ. of California, Los Alamos, N. Mex.

Tritium absorbed by rats from inspired air containing HT or HTO is proportional to the tritium activity in the inspired air. When present as HTO a very high percentage of the tritium activity inspired is taken into the body fluids, indicating rapid exchange and absorption of activity across respiratory membranes. If present as HT in inspired air only about 0.05% of the inspired activity appears in the body as HTO. Apparently, biological catalyzation of HT to HTO takes place in the body. The activities studied were not high enough for the absorption of HT per se in body fluids to be detected. HTO ingested by man in 200 cc. of water was completely absorbed in about 45 minutes. Absorption is linear with time. Absorbed tritium dilutes with 57 to 68% of the body weight, suggesting that body water is primarily involved. Tritium elimination from the body in both rats and man is an exponential function of total water turnover. The reservoir calculated from the rate constant is again 57 to 68% of the body weight. The biological half-life of HTO in 9 human subjects varied from 9 to 14 days on *ad libitum* water intake and was reduced to 2½ days in one subject on high water intake. The biological half-life in rats ranged from 4 to 5 days on *ad libitum* water intake. Tritium activity in excreted sweat, insensible perspiration, expired water vapor, sputum, and urine is essentially the same as that in the blood.

*Maze performance in the white rat following physiological recovery from DFP injections.* CHARLES E. PLATT AND DELOS D. WICKENS (introduced by DAVID B. DILL). Ohio State Univ., Columbus.

Di-isopropyl fluorophosphate is an anti-cholinesterase agent which has a pronounced effect on the functioning of the nervous system through its action in destroying the enzyme cholinesterase. It is of interest to determine if there are persistent effects of DFP which may affect behavior but which are not revealed by chemical analysis of brain tissue. This study was designed to investigate the effect of injections of DFP on maze learning by rats, following physiological recovery from the effects of the drug. Sixty male albino rats were used. All animals were the same strain and were approximately 60 days old at the beginning of the experiment. Thirty-four randomly selected animals were injected subcutaneously with 2 mg/kg. of DFP in a suitable vehicle. The 26 animals of the control group were injected with similar quantities of the vehicle. Eleven weeks after injection, all rats were placed on 22-hour food deprivation and were trained on a 14-unit multiple-T alley maze. Two trials per day were given until the criterion of 3 errorless trials out of 4 was attained. Analysis of data in terms of the mean number of trials required to reach the criterion by the 2 groups, revealed a *t* ratio of 1.19 with a *P* value of between the 20% and 30% levels. It cannot be stated with any high degree of confidence that, under the con-

ditions of this study, there was any persistent effect of the DFP which was revealed in maze learning.

*Mutual advantages of affiliation between armed forces laboratories and civilian academic institutions.* Captain JOHN R. POPPEN, MC, U.S.N. Aeromedical Acceleration Lab., Naval Air Development Center, Johnsville, Pa.

Although Armed Services laboratories are well equipped with facilities for research and development, they are recurrently criticized for being meagerly staffed and inadequately affiliated with civilian institutions. One reason often advanced for the paucity of staffs is the fact that they offer limited appeal to scientists principally because they are not engaged in the type of research which produces findings worthy of publication leading to wider and increased academic recognition. Occupation in Service laboratories under Civil Service appointment is considered to present limited and poor opportunity for advancement in the field of science. This need not be so. Agreed affiliation between a Service laboratory and a university can be established to mutual advantage. Critical features of this affiliation should include the following: Appointment of selected civilian personnel of the laboratory to appropriate position on the Faculty with attendant responsibility in the form of research guidance, seminars, teaching etc. Additional compensation for this responsibility can be arranged. Members of the Faculty and other investigators should be permitted to conduct research in the Service laboratory into studies mutually agreeable to the two institutions and consonant with the mission of the laboratory. Civilian and military personnel of the laboratory should be permitted to enroll in the university and pursue studies leading to advanced degree credit. Dissertations on theses conducted in the Service laboratory should be recognized by the university as valid for degree consideration. Mutual working agreements between the Faculty and the Director of the laboratory should be established. The integrated cooperation resulting from an agreement of this nature will provide recognition of the efforts and worth of Civil Service appointees with promise of advancement either within the Service or appointment to the faculties of civilian academic institutions. The results of research conducted in Service laboratories will be more widely accepted as of scientific value and there will be accelerated advance to mutual advantage.

*Sodium cyanide on mammalian nerve.* E. L. PORTER, L. BURNETT\* AND E. N. WILSON.\* Med. Branch, Univ. of Texas, Galveston.

Porter and Wharton (*J. Neurophysiol.* 12: 109, 1949) reported that shutting off the blood supply to a cat nerve resulted in a heightened irritability of the nerve lasting many minutes. Question arose as to whether this might have been due to changes in the conditions of stimulation instead of ischemia. Artery and vein are

both clamped in this experiment. If it chanced that the vein were occluded and not the artery the nerve would tend to swell and make an initial strength of stimulus more effective than before, thus giving the appearance of an increase in irritability. To test this, the effect of anoxia on the nerve has been tried by leaving blood vessels intact and surrounding the nerve at the point of stimulation with a weak solution of sodium cyanide. A solution of 0.001% results in marked increase in irritability of the nerve. If the cyanide be replaced by Ringer solution, the nerve returns to its original irritability after perhaps half an hour and the experiment may be immediately repeated. This experiment confirms the view that ischemic loss of oxygen results in heightened irritability of mammalian nerve. Further confirmation of the fact is obtained by sectioning artery and vein to the nerve. Here shrinkage of the nerve would occur rendering the initial strength of shock seemingly less effective. But the initial shock on the contrary is now more effective just as in the case of clamping the blood vessels.

*Bronchoconstrictor effect of thiamine.* MELVIN POST AND JAY A. SMITH (introduced by PIERO P. FOA). Chicago Med. School, Chicago, Ill.

Death and toxic effects of moderate doses of thiamine have been reported in human patients. It was thought that thiamine, like other curare preparations, might liberate histamine and produce bronchoconstriction. Accordingly, dogs were anesthetized with Nembutal, spontaneous respiration suppressed by additional Nembutal, by injection of alcohol into the cisterna magna, or by pithing the brain. Artificial respiration was maintained at constant rate and pressure and applied to the intrathoracic space; intratracheal pressure was recorded and used as an index of bronchial caliber. Blood pressure, electrocardiogram, and toe twitch were recorded. It was established that when blood pressure was high, and, by implication, sympathetic tone was likewise high, thiamine always caused some bronchoconstriction, and, in addition, hypotension, bradycardia, and neuromuscular block. Some of this bronchoconstriction could be blocked by benedryl. When blood pressure was low, and, by implication, sympathetic tone was likewise low, thiamine caused exceedingly slight bronchoconstriction. The lack of marked bronchoconstriction was not due to faulty apparatus nor insensitivity to histamine, because histamine produced marked bronchoconstriction. A few experiments with intocotrast showed that its action paralleled that of thiamine. It is concluded that thiamine causes bronchoconstriction, as does curare, in two ways: first, innocuous bronchoconstriction due to ganglionic blockade, and second, in susceptible animals, bronchoconstriction of considerable magnitude; since the latter type can be blocked by benedryl, it is probably due to liberation of histamine. Thus the deaths and toxic effects of thiamine reported

in human patients may be due to liberation of histamine in susceptible patients.

*Human electromyogram.* H. J. RALSTON, V. T. INMAN, B. FEINSTEIN AND B. LIBET. College of Physicians and Surgeons, and Univ. of California Med. School, San Francisco.

Studies on isolated voluntary muscles in cineplastic amputees, and on muscles of normal subjects, yield the following results: 1) In a muscle kept at fixed length, the integrated EMG parallels the isometric tension with varying degrees of effort. 2) When the muscle is allowed to change in length, no parallelism exists between isometric tension and EMG. 3) The EMG of large muscles characteristically exhibits a reduction in amplitude as the muscle length is increased. This is probably due to cancellation of potentials resulting from desynchronization. 4) No regular relationship between power developed by muscle and EMG amplitude exists.

*Relations between cutaneous blood flow and sweating at various environmental temperatures.* WALTER C. RANDALL, A. B. HERTZMAN AND H. E. EDERSTROM. Dept. of Physiology, St. Louis Univ. School of Medicine, St. Louis, Mo.

Nude healthy young men were exposed for 3 to 4 hours at 5 ranges of temperature between 23° and 38°C. while sweating rates (by desiccating capsules) and blood flow (calculated from thermal conductances as well as by the photoelectric plethysmograph) were simultaneously determined on 10 different areas of the body. Water losses from non-sweating skin revealed much lower values than those generally accepted for the water of diffusion, being lowest on the extremities and trunk, and higher on the head, palmar and plantar surfaces. Marked differences in sweating rates were observed on different skin areas. Although the palms show relatively high water losses even at low air temperatures, thermal sweating appears first and most profusely on the calf and thigh, next on the lower trunk and forehead, and finally on the upper extremities. Some discrepancies in blood flow as calculated from thermal conductances and from the amplitude of the volume pulse were observed, but it is thought that these would be greatly reduced if known errors in the thermal conductance method could be corrected. Reasonable parallelism between sweating and blood flow responses were generally demonstrated with rising air temperatures, but significant discrepancies sometimes appeared on different skin areas, thus failing to support the impression that in the zone of vasomotor regulation there is a smoothly operating decrease in vascular tone over the entire skin surface complementing the onset of generalized sweating. The danger of inferring vascular and sweating responses on the body as a whole from observations limited to the hands and feet or any other limited areas is illustrated.

*Measurement of volume flow through that circulatory bed with shortest circulation time.* W. J. RASHKIND (introduced by H. C. BAZETT). Dept. of Physiology, School of Medicine, Univ. of Pennsylvania, Philadelphia.

The arterial time-concentration curves of dye injected into the right auricle of dogs show several consecutive concentration plateaus. The injection is maintained at a constant rate throughout the duration of the procedure and samples are collected at rates of 2/second or 3 every 2 seconds. The first plateau represents the average initial dilution of dye (Stewart) and has been shown to be a function of cardiac output. It is postulated that the secondary plateaus are due to recirculation through functionally discrete circulatory beds. In a series of experiments on 16 dogs the volume flow through the first recirculatory bed (FRB) was derived and is expressed here as percentage of total cardiac output. Preliminary experiments under Nembutal anesthesia with pulse rates of 120-150 showed a mean cardiac output of 2667 cc. with a value for FRB of 17.1 per cent on the average. Subsequent studies on dogs anesthetized with morphine, dial-urethane and Nembutal mixtures (pulse rates 70-100) had a mean cardiac output of 2245 cc. and an average FRB value of 9.3%. Three determinations were done on an unanesthetized, trained dog. The mean cardiac output was 2051 cc. and the mean FRB 6.8%. The pulse rate ranged between 60-72. The dog was subsequently anesthetized with Nembutal and the pulse rate increased to 120, the cardiac output to 2933 cc., and the FRB to 14.1%. The 6.8% average value for the FRB in the unanesthetized animal suggests that this figure may be related to the coronary blood flow. This relationship is supported by the recirculation times of 12-16 seconds in these studies, and by anatomical considerations.

*Effect of prolonged administration of cortisone and ACTH on synovial potentials in rheumatoid arthritis.* C. I. REED, I. E. STECK, M. M. MONTGOMERY AND NORMAN R. JOSEPH. Univ. of Illinois, Chicago Professional Colleges, Chicago.

It has been shown that the high positive potentials in synovial structures in rheumatoid arthritis are reduced by the intramuscular injection of 25 mg. of ACTH or 100 mg. of cortisone. In the case of the first, the response appeared in 5 to 20 minutes, and the level sometimes was depressed to zero within a few hours. Within a week, on prolonged administration of 25 mg. every 6 hours, the resting level returned to that previously seen and in some instances even higher. Cortisone gave a less abrupt depression, the lowest level being reached only after 2 to 3 days. Usually within 10 to 14 days the initial level was restored. Even after the subjects had reached a resting level comparable to that seen initially, they responded in a manner



comparable to that seen at first, but the depression was not so great and was less prolonged.

*Technique for study of autonomic (cardiac, respiratory, PGR) and motor adaptive responses (CRS) in the human.* WILLIAM G. REESE, W. HORSLEY GANTT and CHARLES STRAHAN. Veterans Administration Hospital, Perry Point, Md.

A conditional reflex technique for psychophysiological study of the normal and the abnormal human is described. The subject is subjected to Gantt's standardized test of ability to form CR's, with simultaneous parallel studies of whole and of part responses and their interrelationships. Earlier studies of emotions (CANNON *et al.*) were confined chiefly to unconditional responses; and previous CR studies of motor adaptive responses excluded visceromotor components. The present approach stresses conditional autonomic responses and emotional concomitants of somatic responses. Illustrative examples of the diagnostic use of the method are given. Suitable apparatus utilizes semi-automatic signaling devices and polygraphic recording of respiratory movements, hand movements, heart rate, blood pressure, psychogalvanic reflex, accompanying learned responses.

*Electrical resistance of the resting and secreting stomach.*

WARREN S. REHM. Dept. of Physiology, Univ. of Louisville, Louisville, Ky.

The direct current electrical resistance of the anesthetized (amylal) dog's stomach was determined by measuring the potential difference across the stomach before, during, and after the sending of small direct currents (20-60 microamperes/cm.<sup>2</sup>) across the stomach in both directions. A double chamber technique similar to the one described previously (*Am. J. Physiol.* 144: 115, 1945) was used. One pair of electrodes was used for current sending and another pair for P.D. measurements. The temperature of the chamber was maintained to within  $\frac{1}{4}^{\circ}\text{C}$ . of the rectal temperature. The resistance between the two P.D. electrodes is equal to the P.D. during current flow minus the P.D. before (or after) current flow divided by the current. The resistance of the stomach is equal to the above-measured resistance minus the resistance of the fluid (0.16 M NaCl) between the stomach and the P.D. electrodes. The average specific resistance (resistance/cm.<sup>2</sup>) of the resting stomach (5 experiments) was 454 ohms, and this decreased by 33% to 306 ohms during secretion of HCl. Secretion was stimulated with histamine and mecholyl. Following the cessation of secretion the specific resistance increased to approximately its original level. Interruption of the blood supply resulted in an increase in the resistance, which reached a maximum in slightly less than one hour, of approximately two times the resistance of the resting stomach. The resistance then gradually decreased to approximately the level of the secreting stomach within 12 hours.

*Newly modified electromagnetic flowmeter* (Demonstration). ALFRED W. RICHARDSON, ADAM B. DENISON\* and HAROLD D. GREEN. Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

This instrument is a newly developed A.C. electromagnetic blood flow meter built for the purpose of measuring pulsatile and mean blood flow. The device is a modification of the original cannula pickup of Richardson, Randall and Hines. The new features include a plastic housing, flexible cannula tips, and a new grounding method within the cannula using conductive plastic. These new additions make the instrument more adaptable to physiologic conditions of flow measurement. Also, the amplifier features a newly designed A. C. circuit which allows manual control of zero alignment and an improved system of canceling background voltages. It employs a 60-cycle/second carrier wave which when fed into a phase discriminator accurately measures backflow up to 100% of forward flow with less than 5% error in either direction, a feature not incorporated in the original instrument. Full deflection may be obtained on an Esterline-Angus 5 m.a. recorder with 50 ml/minute flow or a Brush record with a 250 ml/minute flow. Adjustments allow other amplifications of less gain.

*Politary patterns for chest leads.* JANE SANDS ROBB.

Dept. of Pharmacology, New York State College of Medicine, Syracuse.

Thoraces of normal young men have been mapped into a grid and numerous 'unipolar' electrocardiograms recorded, even as many as 72. Galvanometer connections for the entire series were maintained at the convention for wiring always accepted for leads 1, 2 and 3. Over some areas on each thorax, simultaneously recorded leads present an initial upstroke and over other areas an initial downstroke, signalling the onset of the ventricular complex. A smoothly curved line can be drawn separating these two types of wave direction. Anatomical and physiological reasons are presented to establish that upstrokes and also downstrokes are caused by potentials from limited areas, and that the reason for bidirectional initial waves depends only on whether a particular depolarization wave is traveling toward or receding from a particular electrode. It is shown that these approaching and receding waves form a demarcation pattern characteristic of the habitus of the individual. The normal demarcation pattern for any individual provides a vivid portrayal of the heart's electrical activity as projected to the thoracic wall. An individual, with an old infarction, has a demarcation pattern wholly unlike that of any normal students. When the individual is of intermediate type (neither sthenic or asthenic) the demarcation lines between positive and negative initial waves, positive and negative P waves, and positive and negative T waves may be almost identical in contour and place-

ment. When the heart is either vertical or horizontal especially when combined with rotation the three demarcation lines have dissimilar patterns. In the presence of the infarction noted above, the T demarcation line is more altered than are those for the initial deflection ('R' or 'Q') or for 'P'. Successive lesions result in progressive shifts of these demarcation lines. Studies on abnormal patients as well as follow up studies in normals should be made to determine whether such study gives sufficient additional information to balance the time and expense involved in recording from so many points.

*Effect of various antioxidants on action of epinephrine in man.* CHARLOTTE ROBERTSON\*, JOSEPH LASH AND M. I. GROSSMAN. Univ. of Illinois College of Medicine, Chicago.

Richards in 1943 reported that sodium bisulfite increased the toxicity of epinephrine if these two compounds were injected subcutaneously or intramuscularly at the same site, but when given intravenously or injected at different sites no increase in toxicity, as measured by lethal doses, was noted. We sought to determine if this 'bisulfite phenomenon', which presumably acts by increasing the rate of absorption of epinephrine, also occurs in man in physiological doses. We compared the action of epinephrine HCl solution on blood pressure and pulse rate with and without sodium bisulfite. In the experiments without bisulfite, sodium hypophosphite was used as an anti-oxidant. In 13 male subjects two blood pressure and pulse rate determinations were taken every 10 minutes for 20 minutes before, and one hour after, injection. One cc of 1 to 1,000 epinephrine with 0.1% sodium bisulfite was compared with the same concentration of epinephrine with 0.5% sodium hypophosphite given subcutaneously. The systolic pressure with epinephrine containing bisulfite rose to 151 mm. Hg whereas with the epinephrine with hypophosphite it rose slowly to 133.5 mm. Hg. To establish the differences in the peaks of action the experiments were repeated on 6 other subjects and the blood pressures and pulse rates were followed until they returned to normal. The absolute increase in systolic pressure with bisulfite was 41.7 mm. Hg. with the peak at 14.3 minutes, and the increase with hypophosphite was 20.7 mm. Hg. with the peak at 56.7 minutes. The diastolic pressures were decreased with both drugs. The differences in pulse rates were not statistically significant.

*Bronchiolar muscular tone: a significant factor in control of pulmonary circulation.* SIMON ROBBARD. Cardiovascular Dept., Med. Research Institute, Michael Reese Hospital, Chicago, Ill.

Comparative analysis of the pulmonary circulation shows the normal pulmonary arterial pressure to be 25/10 mm. Hg. in mammals, birds and reptiles. Interposition of the lung circuit between the two ventricles

would appear to require control mechanisms adequate to equalize the ventricular outputs, prevent pulmonary engorgement and transudation (edema), while affording optimal conditions for gas exchange. Nevertheless, the pulmonary vessels have been shown to have minimal neurogenic or pharmacodynamic control. As a result, the lungs have been considered a passive vascular bed controlled primarily by the balance of the output of the two ventricles. Our studies on the pulmonary arterial pressure in dogs may be interpreted as showing that the pulmonary circulation is under active control. The lungs have within them a well developed muscular apparatus which apparently controls pulmonary hemodynamics indirectly: the bronchiolar musculature. During inspiration, the bronchioles dilate actively, and intra-alveolar pressure falls. An index of this intra-alveolar pressure is seen in the pulmonary diastolic pressure. Adrenergic influences also dilate the bronchioles, reducing intra-alveolar and pulmonary diastolic arterial pressures. If this effect is marked and prolonged, pulmonary congestion and edema may ensue as a result of the inhibition of the protective bronchiolar muscular tone. During expiration, vagotonic influences constrict the bronchioles, increasing resistance to outflow of air from the alveoli and thus increasing the intra-alveolar pressure. This increased intra-alveolar pressure compresses the pulmonary capillaries, reducing their volume, and acts to force transudates back into the vessels. Histamine produces an emphysematoid expiratory bronchiolar spasm, with increased intra-alveolar and pulmonary diastolic pressures but with a decreased tendency to transudation. The bronchiolar muscle is thus seen as an important factor in the regulation of the pulmonary circulation.

*A model demonstrating cardiovascular dynamics (Demonstration).* SIMON ROBBARD. Cardiovascular Dept., Med. Research Institute, Michael Reese Hospital Chicago, Ill.

A model of the circulation which graphically demonstrates hydrodynamics has been designed and used with success in teaching, and in analysis of problems in cardiovascular hemodynamics. Ventricles composed of latex rubber fill on the basis of atrial pressure and operate in accordance with Starling's law. Energy for ventricular contraction is supplied by air pressure to the outer ventricular wall. Limitation of energy produces ventricular dilatation and failure when the work load is increased. Phasic adjustments of rate, strength of contraction and duration of systole can be made with valves operated by a windshield wiper motor. Valves are made of glass cones in which a stainless steel ball moves. The range of motion of the ball can be quantitatively limited by means of stylets which prevent the ball from seating (insufficiency) or opening (stenosis). Heart sounds bearing some resemblance to normal and pathological sounds are produced. Simultaneous atrial, intraventricular, arterial and venous

Pressure pulses may be observed in glass manometers. A lung model is interposed between the right and left ventricles to demonstrate effects due to position, stasis, congestion and edema. Arterial elasticity is introduced by a segment of expansible rubber tubing in the arterial tree. The systemic circulation is composed of three alternate channels for arterial outflow, representing a preferential channel with little vasomotor tone, one with variable tone and a blood reservoir. Flow in any part of the model may be measured with stromuhrs. The hydrodynamics of interatrial and interventricular septal defects and various shunts including patent ductus arteriosus are demonstrated. Attachments include apparatus for producing atrial contraction and pericardial constriction.

*Determination of water vapor pressure of respired air by electro-chemical means.* JOHN T. RODGERS (introduced by FRED A. HITCHCOCK). Dept. of Physiology, Ohio State Univ., Columbus.

Humidity sensing elements consisting of a plastic cylinder with a dual winding of wire covered by a hygroscopic film of dried lithium chloride or dried phosphoric acid were used to determine water vapor pressure. The electrical resistance of the hygroscopic film varies with microchanges in moisture content of surrounding air. They were calibrated by placing in known water vapor pressures. Measurement of human alveolar water vapor pressure showed values ranging from 42 mm. Hg. at 23°C room temperature to 46 mm. Hg. at 35°C. Measurement of water vapor pressure in two cases with subjective symptoms of dry throat gave values of 41.5 mm. Hg. Hyperventilation for 15 seconds lowered water vapor pressure of alveolar air by 9 to 14 mm. Hg. Exhalations containing cigarette smoke showed values 1 to 5 mm. Hg. lower than normal; return to normal occurred with one to four respirations. Elements housed in wire cages offering little resistance to air flow were placed down the trachea of anesthetized dogs and continuous records were made. These showed that a lowering of water vapor pressure occurred with each inspiration. Alveolar water vapor pressure was higher with a lower rate of respiration. Exposure to cold lowered the pressure. The total amount of water lost via respiration has been determined with the aid of these elements by collecting expired air in a Douglas bag and measuring the water vapor pressure at a known elevated temperature. These elements have also been used to measure the private climate of the body under various atmospheric and physiological conditions.

*Effects of anesthesia on refractory period of auditory cortex.* MARK R. ROSENZWEIG. Psycho-Acoustic Lab., Harvard Univ., Cambridge, Mass.

In studying the electrophysiological responses of the auditory cortex to successive acoustic stimuli, anesthetic level is an important parameter. Although the

response to a single stimulus may not change greatly over a wide range of anesthetic levels, the recovery function changes markedly. The amplitude of the response to the second of two click stimuli was plotted as a function of the time-interval between the two clicks. Typically the recovery function showed a few damped cycles of increasing and decreasing amplitude before coming to rest at the level of complete recovery. Altering the anesthetic level changed both the periodicity of the recovery function and the degree of recovery attained during the first cycle. With light barbiturate anesthesia, the peak of the first cycle of recovery might occur within a time-interval of about 50 milliseconds, and the peak often showed supernormality. As the depth of anesthesia was increased, successively greater intervals were required for recovery, and the initial peak was subnormal. With deep anesthesia recovery required over 200 milliseconds and the function was no longer cyclical. Slides were shown to illustrate the sensitivity of the recovery function to changes in anesthetic level. During the depression of the response to the second stimulus there was an increase in the duration of its surface-positive deflection, especially with light anesthesia. This increase is due in part to the fact that the negative components of the response show longer refractory periods than does the positive component. When secondary cortical responses followed the evoked response to the first click, they showed the same periodicity as the recovery function. Often the secondary responses had the dimensions of alpha waves; sometimes they appeared as repetitive spike responses. In either case, the evoked response to the second click increased when it occurred on the rising phase of the secondary response and decreased when it occurred on the falling phase.

*Absorption of phosphate from subarachnoid space.* JACOB SACKS AND GEORGE G. CULBRETH\*. Biology Dept., Brookhaven Natl. Lab., Upton, L.I., N. Y., and Division of Neurological Surgery, Johns Hopkins Hospital, Baltimore, Md.

A comparison has been made of the time-courses of the relative specific activities (rsa) of the plasma inorganic phosphate in cats for the first 4 hours after intravenous and intracisternal injection of tracer phosphate, and of the rsa of the inorganic, phosphocreatine and ATP-P of brain and muscle at the end of this period. The highest rsa of the plasma phosphate following the intracisternal injection was found in samples of carotid blood taken 30 minutes after the injection. The subsequent course of the rsa indicates that approximately two-fifths of the tracer material injected does not appear in the plasma within the 4-hour period. The rsa of the P fractions of the brain in these animals was generally higher than that of the final plasma samples. The rsa of the phosphocreatine and ATP-P of the muscles of the animals which received the tracer intracisternally was about two-fifths as great

as in the animals in which the tracer was injected intravenously. The  $\text{r}_{\text{sa}}$  of these compounds in the brain was significantly higher than in the muscles of the animals in which the tracer was injected intravenously. (The radioactive phosphorus used in these experiments was supplied by the Isotopes Division of the Atomic Energy Commission. This research was carried out under the auspices of the Atomic Energy Commission.)

*Defense interrelationships between vitamin A and reticulo-endothelial system (RES).* STELIOS C. SAMARAS, NICHOLAS DIETZ, JR., LEO CLEMENTS and MICHELE GERUNDO (introduced by CHARLES M. WILHELMJ). Depts. of Biological Chemistry and Nutrition, Anatomy and Pathology, Creighton Univ. School of Medicine, Omaha, Nebr.

To elucidate the question of the existence of a defense unity between RES and vitamin A in combating infections, 100 male white rats, 4 weeks old, were used. Several groups were put on a vitamin A-deficient diet, to develop avitaminosis A, and compared with controls. Control rats on standard Purina diet, and rats on vitamin A-deficient diet, plus carotene, appeared normal. The experimental animals presented the characteristic weight loss, weakness, eye symptoms and bronchopneumonia. We injected all animals subcutaneously with the same amount of colloidal carbon (Higgins India Ink), to determine their reaction toward a foreign substance. The avitaminotic rats exhibited overnight an aggravation of their condition until they were almost blind. Histological examination of all organs showed the following: in normal rats, there was widespread phagocytosis of carbon; in avitaminotic rats, phagocytosis of carbon was practically absent; in hypovitaminotic rats and avitaminotic rats given carotene, there was some phagocytosis, especially in the liver and lungs. A certain amount of hemorrhage in the lungs of avitaminotic rats indicated increased capillary fragility. Conclusions: the hypothesis that the RES is the biochemical factory of conversion of provitamins to vitamin A and also the storehouse whence the animal organism mobilizes these substances as anti-infectious factors is substantiated by our findings. The diminished amounts of vitamin A found after infections may be due to the decreased production of vitamin A because of preoccupation of the RES in the fight against the invading microorganisms, and because of an increased vitamin A demand for immunochemical reactions.

*Phosphate metabolism ( $P^{32}$ ) of resting and stimulated nerve.* ARTHUR J. SAMUELS (introduced by R. W. GERARD). Dept. of Physiology, Univ. of Chicago, Chicago, Ill.

Frog sciatics stimulated *in vitro* were earlier shown to decrease their ATP and CrP content as compared to resting controls. No increased disappearance of carbohydrate occurred, but a slight increase of acid soluble

P suggested a possible phospholipin fuel for the extra metabolism of activity. Present work, soaking paired nerves in  $P^{32}$  washing, then resting or stimulating, fractionating the P compounds, and determining amounts and specific activities of the fractions, has given further insight into nerve metabolism. In 15 experiments (no tracer), no change of phospholipin content appeared after tetanization of 1 to 12 hours; but a considerable rise in specific activity (3 tracer exper.) suggests an increased turnover. Specific activity of ATP and CrP also rose more rapidly in stimulated nerves (ATP was above CrP both at rest and under stimulation), as did that of 'phosphoprotein'; while specific activity of the glucose 6-P fraction, and probably of the phosphoglyceric acid fraction and of nucleic acid, rose more slowly on stimulation than at rest. Reducing power of the total acid soluble fraction, or of the glycogen fraction, was again found unchanged on stimulation.

*Adaptation of men and animals during prolonged exposure to increased carbon dioxide concentrations.* K. E. SCHAEFER. U. S. Naval Med. Research Lab., New London, Conn.

Previous experiments have shown that exposure of men to 3%  $\text{CO}_2$  in air over a period of 6 and more days produces a biphasic reaction, that is, a period of excitation followed by depression. Tests of letter-canceling and hand steadiness, chronaxie measurements and changes in the EEG pattern confirmed the subjective sensations. Excitability of the respiratory center decreases while alkali reserve of blood increases, mainly due to retention of alkali by the kidneys. Experiments with guinea pigs, rats, mice and dogs using  $\text{CO}_2$  concentrations from 3% up to 24% showed biphasic variations in the following: bodyweight, motor activity, blood sugar, alkali reserve. At lower  $\text{CO}_2$  concentrations around 3% of the excitant phase was not regularly seen. Higher  $\text{CO}_2$  concentrations of 12% or more exhibited both phases distinctly. A combined physiological histological investigation of the endocrine glands, which I conducted at the Physiological Institute in Heidelberg in collaboration with 2 pathologists Drs. Klein and Zinck, revealed more about the nature of the biphasic effect of  $\text{CO}_2$ . Staining and morphological methods as well as adrenaline content determinations and other functional tests proved that during prolonged exposure of guinea pigs and dogs to 3%  $\text{CO}_2$  a hypersecretion of adrenalin is followed by a hyposcretion, the latter due to a diminished synthesis. A hyper- and subsequent hypophase was found too in the adrenal cortex, in the basophilic cells of the pituitary glands (Klein). The thyroids become more and more inactive with prolonged exposure time to 3%  $\text{CO}_2$ .

*Transient electrical characteristics of squid nerve.* OTTO H. SCHMITT. Univ. of Minnesota, Minneapolis.

Measurements of membrane characteristics by the

complex attenuation method in the range 40–10,000 cps on normal squid nerve emphasize the existence of at least 3 separate electrical components, one relatively stable and probably associated with physical structure, a second directly related to ionic permeability, and a third which is highly responsive to physiological state. The stable component is responsible for the familiar 1 mfd/cm<sup>2</sup> capacitance and has a relatively small temperature coefficient. The permeability term is closely associated with excitability but has an unpredicted frequency minimum. The unstable susceptance component is highly temperature sensitive and responds sharply to ionic changes in the environment. Knowledge of these coefficients also facilitates measurement of the volume resistivity of squid axoplasm in the intact axon as correction for the conductivity of solution clinging to the outside of the nerve can be made. Studies of axoplasmic resistivity as a function of temperature, frequency, and fiber size have been made. The mean conductivity value found is 22.5 millimhos cm. at 20°C. or about 51% of the conductivity of Woods Hole sea water. Conductivity changes almost precisely as does that of appropriately diluted sea water in the range 5° to 30°C. No detectable variation of resistivity with frequency is found above 500 cps and a slight measured variation in the 100 cps region is probably a by-product of the rapidly changing membrane impedance in this frequency range. No systematic variation of axoplasmic conductivity with fiber size has been noted.

*Direct measurement of pulse wave velocity in man.*

T. G. SCHNABEL, W. J. RASHKIND AND L. H. PETERSON.  
Dept. of Physiology, Univ. of Pennsylvania, School of Medicine, Philadelphia.

Using small plastic catheters, direct recordings were made of pulse pressure curves in the central and peripheral arteries of 16 normal males, 20 to 40 years of age. Pulse wave velocities were calculated by the time difference between the q wave and the onset of the pulse at various sites in the arterial tree. The time from the q wave of Lead II of the ECG to the start of the pulse at the aortic valve averaged .085 seconds. The average pulse wave velocity in the thoracic and ascending aorta was 5 meters/sec. In the region of the diaphragm and in the subclavian artery there is an apparent deceleration of the pulse wave, the velocities being 4.6 and 4.2 meters/sec. respectively. There is a marked increase in the velocity of the pulse in the brachial artery (8.2 meters/sec.) and in the iliac artery (8.4 meters/sec.). There is no significant change in the mean pressure with a gradual increase in the pulse pressure as one progresses from the aortic valve to the femoral artery.

*Local responses of single medullated fibers in a nerve.*

GORDON M. SCHOEFFLE AND JOSEPH ERLANGER.  
Washington Univ. School of Medicine, St. Louis, Mo.  
By stimulation with rectangular currents, local re-

sponses have been demonstrated in single fibers of the frog phalangeal preparation. In most respects we confirm Hodgkin and Rushton's observations on giant fibers. Reasonably enough, the frog latencies are 30 times as brief while ratio of local to propagated spike height is  $\frac{1}{4}$  as great. The local response appears initially as a gradually increasing nonlinear function of the applied voltage which merges into a discontinuous monophasic hump at stimulating voltages above 80% of rheobase. This latter component, of approximately spike duration, has a falling phase briefer than its rising. Spontaneous variations in excitability allow the responses elicited by threshold currents to range between a full-fledged spike and an almost imperceptible hump. If the hump terminates in an upward inflection it always develops into a fully formed spike. If repetitive responses appear there may be, say, 3 responses, or only 2 or one followed in the latter cases by one or two humps, all spaced by the usual intervals of repetitive responses. Some of these humps seem to be diphasic. Cathodal electrotonic potentials in sheath-free sciatic nerve are initially linear functions of the applied voltage but become nonlinear a few tenths of a msec. after start of the stimulus. It is a component of this total potential that is observed to reach a constant value at threshold, regardless of the time-course through which it is attained.

*Treatment and prophylaxis of experimental renal hypertension with crude hog hypertensinogen.* LIONEL SCHOUR\* AND GEORGE E. WAKERLIN. Dept. of Physiology, Univ. of Illinois College of Medicine, Chicago.

Daily intramuscular injections of crude porcine hypertensinogen in a dose of 1 dog unit/kg. of body weight for 4 months did not prevent the subsequent development of experimental renal hypertension in 4 dogs. Daily intramuscular injections of crude porcine hypertensinogen in a dose of 1 DU/kg. of body weight for 6 months did not significantly affect the blood pressure of 4 chronic renal hypertensive dogs. It was not possible to demonstrate the development of any neutralizing ability (anti-hypertensinogen) against porcine hypertensinogen in the sera of dogs injected with 1 DU of crude porcine hypertensinogen per kilogram of body weight per day for as long as 6 months. In view of the strong evidence now presented by our research group for the antihypertensive effect of antirenin and for the renin-hypertensin concept of the pathogenesis of experimental renal hypertension, further work with purified hog hypertensinogen in larger doses is indicated.

*Evidence for low frequency relaxation mechanism in frog muscle.* HERMAN SCHWAN. Aero Med. Equipment Lab., Naval Base, Philadelphia, Univ. of Pennsylvania, Philadelphia.

Measurements of the electrical impedance of frog

muscle have been performed with a high precision alternating current bridge between 20 and 200,000 cycles. The results prove that the behavior at the very low frequencies cannot be explained simply by the existence of cell membranes with constant phase angle. It is shown that the total impedance is subject to two changes with frequency. One of these dispersion effects is characterized by a characteristic frequency of 60 kc. and the second one by 75 cycles. The characteristic frequencies for capacity and conductivity are both the same in each of the two dispersions. If we plot the reactance against the resistance, we obtain two circles, a large one with its center below the resistance axis and a smaller one at the low frequencies with the center on the resistance axis. The phase angle of the cell membrane, as determined from the high frequency circle, is in agreement with former determinations. The behavior at the frequencies below 100 cycles is in excellent agreement with the assumption of a relaxation mechanism which can be characterized by one single relaxation time. A structure of the membrane could be responsible for this low frequency behavior. Measurement of the impedance of muscle as a function of time shows that the impedance variation of higher frequencies which has been explained by a polarizable cell membrane, starts to change at first after about 2 days and is due to the breakdown of cell membranes. The low frequency change, due to a simple relaxation time effect, however, is subject to change much faster.

*Effect of shock and hemolysis on survival time in dogs.*

WILLIAM SCRUGGS, WILLIAM H. OLSON AND H. NECHELES. Dept. of Gastro-Intestinal Research, Med. Research Institute of Michael Reese Hospital, Chicago, Ill.

Large male dogs were anesthetized with Nembutal, and hemoglobinemia was produced with standardized application of high frequency sonic vibrations. Shock was produced by histamine, with the Blalock crush clamp (traumatic) and by the injection of 10 c.c. of 50% glucose intraperitoneally. All control animals died within 6 to 20 hours from the shock procedure alone, regardless of the type of shock used. Combining hemolysis with the administration of 10 c.c. of 50% glucose intraperitoneally permitted 75% of the animals to survive for more than 4 hours, and 4 animals recovered completely. On the other hand, all animals that received histamine 5 or 10 mg/kg. in oil s.c. plus hemolysis died within 24 hours. The animals that were subjected to traumatic shock plus hemolysis also died within 24 hours. The possible reason for the differences in survival is discussed.

*Interconnections of somatic afferent areas I and II of cerebral cortex of the cat.* W. SENCER (introduced by C. N. WOOLSEY). Dept. of Physiology, Med. School Univ. of Wisconsin, Madison.

In this investigation the interconnections between

somatic afferent areas I and II of the cerebral cortex have been studied by means of electrical stimulation and electrical recording. Experiments were performed on 20 cats under pentobarbital sodium anesthesia. A typical result was to find that a point in area I projected to a field in area II consisting of a focus, where the responses were large, and a fringe, where the responses declined from the maximum of the focus to zero at the margins of the field. The focus of the response area and the point stimulated were then both found to be related to the same cutaneous area. Similar results were obtained when somatic area II was stimulated and recordings were made in somatic area I. Since the responses were not altered significantly by undercutting the cortex they must have been mediated through intracortical structures. Stimulation of somatic areas I and II also resulted in responses in the precruciate motor cortex. Stimulation of points in somatic area I produced maximal responses at symmetrically placed points in the motor area. The interareal connections thus are fairly systematically and precisely arranged and points related to the same parts of the periphery are optimally related to one another. This is a more discrete pattern of interareal connections than that reported up to this time by strychnization and other techniques.

*Regulation of food intake in dogs.* I. SHARE AND M. I. GROSSMAN. Dept. of Clin. Science, Univ. of Illinois College of Medicine, Chicago.

In 3 dogs with esophagostomies duration of sham feeding was correlated with amount of food being given intragastrically. Sham-feeding duration was tested daily just before the daily intragastric feeding, i.e. tests were done 24 hours after the last intragastric feeding at which time the stomach was empty. Commercial dog food (Pard) and milk was used for both sham and intragastric feeding. When a large intragastric feeding (3-4 liters/day) was given for 10 or more days sham feeding stopped entirely on some days in each dog. When the intragastric feeding was  $\frac{1}{2}$  to 1 liter per day (insufficient to maintain body weight) sham-feeding duration rose to 40 to 45 minutes. When a balloon was placed in the stomach and distended with 500 to 750 cc. of water the avidity and duration of sham feeding were always diminished (average 30%). Control experiments with the empty balloon in place showed no diminution. The inhibition produced by the distended balloon was as great as that produced by the same volume of food introduced into the stomach. In 3 dogs with simple gastric fistulas from 33 to 100% of their normal voluntary daily intake of food was inserted into the stomach through the fistula 3 hours after *ad libitum* oral feeding. This was continued daily for varying periods. Thirty-three per cent intragastric feeding resulted in no discernible change in oral intake after 2 weeks; 50% resulted in oral intakes of 46%, 71% and 72% of the control level after 2 weeks; and

125% and 190% were followed by oral intakes of 25% and 21% of control level after 10 and 14 days. Weight gain during the experiments ranged from 0 to 7%. In two experiments oral intake was depressed from 3 to 4 weeks following cessation of intragastric feeding, but in the 5 other experiments oral intake returned to its pre-test level within one week after cessation of intragastric feeding.

*Factors regulating renal blood flow and urine flow following acute changes in renal artery perfusion pressure.* R. E. SHIPLEY AND R. S. STUDY. Lilly Lab. for Clinical Research, Indianapolis General Hospital, Indianapolis, Ind.

\*In anesthetized dogs the perfusion pressure to one kidney was altered for periods of 10 minutes at levels ranging from 20 to over 320 mm. Hg and simultaneous measurements were made of renal blood flow (rotameter), extraction per cent inulin, and urine flow. Extrapolations of the flow-pressure curves intersected the pressure axis at 2-10 mm. Hg, which values corresponded closely with those measured at the renal artery cannula when flow to the kidney through the renal artery was temporarily stopped by a clamp. Under the latter condition collateral communications via capsular vessels appeared to be responsible for most of the back pressure recorded at the renal artery. Values which could be designated as 'yield pressure' were indeterminable. Urine flow, beginning at a perfusion pressure around 60 mm. Hg, increased exponentially until at very high perfusion pressures the flow of urine constituted 60-75% of the glomerular filtration rate. The observation that urine flow increases with increasing perfusion pressure, even in the pressure range in which renal blood flow and filtration rate remained essentially constant, suggests that reabsorption of the filtrate is progressively inhibited by a concomitant increase in renal tissue pressure. The latter is probably elevated as the result of passive distention of the arterial tree with increasing perfusion pressure. Changes in afferent and efferent resistance could not be reliably estimated by applying existing formulas since 1) quantitative values for 'yield pressure' and renal tissue pressure were indeterminable and 2) the existence and relative position of filtration and reabsorption equilibrium points are inconstant.

*Humoral control of pepsin secretion.* G. SLEZAK AND M. I. GROSSMAN. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

The gastric phase of HCl secretion can be initiated by the presence of various foodstuffs in the stomach and by distension stimulus. Much evidence indicates that the pyloric portion of the stomach plays a special role in both mechanisms. The suggestion has been made that a pyloric hormonal mechanism exists for pepsin secretion as well as for acid secretion, Grossman, Woolley and Ivy however have shown that chemical

stimulation of the gastric phase with liver extract gave a fundic secretion, the pepsin concentration and output of which was of the same low order of magnitude as that evoked by histamine stimulation. In this work we have been able to demonstrate that the secretion of a denervated fundic pouch which has been stimulated by distension of an isolated pyloric pouch is similar in composition to that evoked by histamine stimulation. The pepsin concentration and output in gastric secretion stimulated by distension of the pylorus or by injection of histamine is insignificant in comparison to that evoked by injection of parasympathomimetic drugs. The facts indicate that the gastric phase, be it excited by chemical or by mechanical stimuli (which have both been shown to be mediated by humoral mechanisms), stimulates the parietal cells primarily and the zymogenic cells little if at all.

*Studies in uremia. II. Effect of irrigation of isolated intestinal loops on experimental uremia in dogs.* HOWARD SLOAN (introduced by P.P. FOA). Dept. of Physiology and Pharmacology, Chicago Med. School, Chicago, Ill.

Exacerbations of chronic renal insufficiency are treated by bed rest, controlled diet and fluid intake. Positive therapy of this condition, at present, can only be through extra-renal means whereby waste products are eliminated and electrolyte and fluid balance maintained. Using an isotonic solution containing sodium, 154 mEq/l.; calcium, 5 mEq/l.; chloride, 106 mEq/l.; bicarbonate, 28 mEq/l.; lactate, 25 mEq/l.; dextrose, 7 gm/l., fistulae of Thiry-Vella fistulized dogs were irrigated, perfusates collected, and non-protein nitrogen (NPN) and creatinine content determined. Blood NPN and carbon dioxide combining power (CO<sub>2</sub>CP) and weight were determined before and after irrigation, 98.3% and 81.6% of the expected urinary NPN, assuming a protein intake of 0.7 gm/kg., were dialyzed from dogs with normal kidneys. Intravenous injection of creatinine caused a marked increase in creatinine content of the perfusate, indicating permeability to creatinine. Irrigation during uremia, caused by tubular necrosis due to intravenous administration of sodium tetrathionate, resulted in the removal of significant amounts of NPN, lowered blood NPN, and raised CO<sub>2</sub>CP. No weight change occurred. If the solution was made hypotonic or hypertonic, weight was gained or lost, and the perfusate volume was less or greater than the inflow respectively. Results indicate that intestinal irrigation can perform significant portions of the kidneys' work of nitrogen excretion and control of acid-base balance, without the dangers of peritonitis or hemorrhage, which are present in other dialysis procedures. Intestinal irrigation appears to be a promising adjuvant in the treatment of chronic uremia.

*Reactions of isolated surviving pulmonary blood vessels to anoxia and autonomic mediators.* DURWOOD J.

SMITH AND JOSEPH W. COX (introduced by W. O. FENN). Depts. of Medicine and Physiology, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The reactivity of isolated surviving pulmonary arteries and veins of man and the lower animals to anoxia, epinephrine, acetylcholine and histamine has been studied using the angioplethysmographic technique of Smith and Syverton. The pulmonary vessels examined were removed from a human or experimental animal immediately post-mortem and mounted in a simple plethysmograph. The vessels were perfused with a modified Tyrode's solution aerated with 5% CO<sub>2</sub> in oxygen under standard conditions for several hours and constriction or dilation to stimuli recorded. Anoxia was produced by using 5% CO<sub>2</sub> in nitrogen in place of the standard aerating gas mixture. The reactions to the autonomic mediators were determined by injecting test doses of these drugs into the perfusate. Thirty-five pulmonary arteries were studied. Fifty-one pulmonary veins were examined. Anoxia for periods up to 40 minutes produced no vasoconstriction of the vessels examined. There is a marked species difference in the reactions of the pulmonary veins. The vasoconstriction of the pulmonary veins to histamine is of interest in view of the marked increase in the blood histamine level which has been reported in hypoxia (see BURKHART *et al.* *Federation Proc.* 8: 19, 1949). The blood histamine levels reported are probably sufficient to cause pulmonary venous constriction in the intact animal.

*Effect of k-strophanthoside on oxygen consumption of embryonic chick hearts as measured in cartesian diver.*

JAY A. SMITH AND MELVIN POST (introduced by PIERO P. FOA). Chicago Med. School, 710 S. Wolcott Ave., Chicago, Ill.

Divers similar to but larger than those described by Claff and Tahmisan (*J. Biol. Chem.* 179: 577, 1949) were used. A cap was placed over the mouth of each diver to slow the loss of oxygen by diffusion and to prevent the loss of part of the oil seal which frequently broke apart during mixing. Hearts from chicken eggs incubated 5 days were used. After an initial observation period of the control divers, Ringer's solution was forced from the neck of the diver by positive pressure, mixing with Ringer's containing the heart; in experimental divers Strophosid (k-strophanthoside, Sandoz) in Ringer's was similarly mixed with Ringer's containing the heart. Strophosid,  $1 \times 10^{-6}$  M, caused no change in oxygen consumption compared to controls but did produce an immediate increase in the heart rate followed by a pronounced slowing; the rhythm was not otherwise affected. Strophosid,  $1 \times 10^{-6}$  M, caused an increase in oxygen consumption of 4.5% above the controls; it produced an immediate increase in the rate, followed by further increase in rate with irregularities, principally extrasystoles from the pacemaker

or auricle, followed, in turn, by partial, then complete heart block. Since the oxygen consumption seems independent of rate and quality of the beat, the increase in oxygen is thought due to Strophosid itself. Since the increase in oxygen consumption occurs with Strophosid in concentration less than that producing minimum irregularities in rate and rhythm, it is thought that the increased consumption is not related to the therapeutic effects of Strophosid.

*Preliminary observations on electrical activity and 'oxygen tension' of brain during 'hyperoxic convulsions.'*

R. R. SONNENSCHN, S. N. STEIN,\* P. L. PEROT, JR.\* AND ELLEN RIDLEY.\* Dept. of Psychiatry, Ill. Neuropsychiatric Institute, Univ. of Illinois College of Medicine, Chicago.

The oxygen tension of the cerebral cortex measured polarimetrically has been recorded synchronously with the electrical activity of cerebrum and cerebellum and with lead II electrocardiogram in more than 50 cats paralyzed with dihydrobetaerythroidin hydrobromide and ventilated automatically with pure oxygen at the tidal volume of about 35 cc. While the ambient pressure of nitrogen was raised to and maintained at 75-pound gauge the oxygen tension of the cortex rose at first slowly then rapidly, typical seizures developed in the cortex and the oxygen tension fell although the ambient and respiratory pressures were maintained.

*Respiratory variations of O<sub>2</sub> and CO<sub>2</sub> content of pulmonary vein blood.* RALPH W. STACY, BUFORD H. BURCH AND FRED A. HITCHCOCK. Lab. of Aviation Physiology and Medicine, Ohio State Univ., Columbus.

Further observations on the variability of the alveolar gas tensions during single respiratory cycles have been made. If such variations (indicated by mass spectrometer experiments) actually occur, they should affect the pO<sub>2</sub> and pCO<sub>2</sub> of blood leaving the lungs. Because of the dissociation curves for O<sub>2</sub> and CO<sub>2</sub>, the O<sub>2</sub> content of the blood should not vary significantly, but the CO<sub>2</sub> content variation should be easily discernible. A technique was devised for the sampling of blood from the pulmonary vein a short distance before its entry into the auricle. These experiments were performed with the chest open. Samples were taken during the first to fourth seconds and during the eighth to eleventh seconds following lung inflation. Throughout this time, the lung volume was constant. Such experiments were made on 7 adult dogs. Van Slyke analyses of the samples showed a greater CO<sub>2</sub> content of the second sample in every experiment. The mean increase was 3.2 volumes %. O<sub>2</sub> analyses showed insignificant changes (mean = 0.2 volumes %). Thus, it is again demonstrated that variation of alveolar gas tensions does occur over single respiratory cycles. Previous attempts to note such variation probably failed because sampling was from the systemic arterial system, and mixing had occurred so that no fluctuations were detectable.



*Plasma cholesterol phospholipid ratio and atherosclerosis in the chick.* J. STAMLER AND L. N. KATZ (with the technical assistance of R. PICK AND C. BOLENE). Cardiovascular Dept., Med. Research Institute, Michael Reese Hospital, Chicago, Ill.

Several recent studies on human atherosclerosis have emphasized the concept that an increase in the plasma ratio, total cholesterol/phospholipid may be a decisive factor in atherogenesis. This report deals with our findings in a study designed to test this hypothesis experimentally in the chick. Two groups of one-day old cockerels were fed a mash enriched with 2% cholesterol plus 5% cotton seed oil. In addition, one of these groups (Group II) received a daily injection of estrogen. Cholesterol feeding resulted in a hypercholesterolemia with a 3- to 5-fold increase in the total cholesterol/phospholipid ratio. The estrogen dosage used did not cause any significant further increase in plasma total cholesterol concentration. Thus the mean plasma total cholesterol levels for the duration of the experiment were: Group I, 732 mg.% (range: 379-1008); Group II, 804 mg.% (range: 443-1264) (normal: 100 mg.%). Since estrogens induce a hyperphospholipemia in chicks far in excess of hypercholesterolemia, the birds given estrogen plus cholesterol (Group II) had a greater hyperphospholipemia (39 vs. 15 mg.% lipid P). Hence the estrogen-treated chicks had a significantly lower plasma ratio, total cholesterol/lipid P (21 vs. 48). The former value tends to approach the normal for the chick (13-14). After 12 weeks, 100% of birds in both groups had gross atherosclerosis of the aorta. Lesions were graded more severe in the chicks receiving estrogen plus cholesterol. Under these experimental conditions, therefore, a reduction toward normal of the plasma ratio, total cholesterol/phospholipid, failed to prevent or retard atherogenesis in the chick.

*Brain stem management of EEG activation.* T. E. STARZL AND C. W. TAYLOR (introduced by H. W. MAGOUN). Northwestern Univ. Med. School, Chicago Ill.

In 1949, Moruzzi and Magoun described a cephalically directed brain stem system whose direct high-frequency stimulation desynchronized electrical cortical activity in a manner simulating that observed in sleep, or in the EEG arousal reaction. At that time the questions of how activating influences were mediated through diencephalic levels to the cortex, and how peripheral stimuli could be discharging into this activation system, were left open. These problems were studied in cats, under B-erythroidine, using a Goodwin stimulator, and recording with a Grass model III amplifier and inkwriter. The diencephalic mediation of the arousal reaction was studied both with stimulating and recording techniques. The forebrain was explored with high-frequency stimulation to determine what regions desynchronized the electrocorticogram. Next

the caudal portion of the activation system was stimulated at high frequencies or with single shocks and bipolar concentric electrodes used to explore for subcortical arousal or evoked potentials. With all three approaches the pathways of the activation system were seen to pass through the midbrain tegmentum, into the basal diencephalon from where its influences reached the cortex thalamically or extrathalamically. The duality of diencephalic mediation was confirmed by acute lesions. Next the distribution in the brain stem of auditory and somatic collaterals evoked by click and sciatic stimulation was studied. In both cases potentials were found widely through the areas of the activation system. By appropriate lesions it was determined that for both modalities an extensive branch of medial collateral fibers occurs through most of the brain stem from the medulla to the posterior thalamus.

*Chemical composition of flatus in man at various altitudes.* F. R. STEGGERDA, W. C. CLARK\* AND I. E. DANHOFF.\* Dept. of Physiology, Univ. of Illinois, Urbana.

To test the effects of altitude on the CO<sub>2</sub> and O<sub>2</sub> concentration of flatus in man, one end of an open-tipped catheter tube was inserted into the previously emptied colon 6 to 8 inches beyond the anal sphincter. The other end of the tube was attached to a T tube, one arm of which afforded a means for the injection of air or collection of flatus; the other arm was attached to an electronic air recording pressure unit outside of the decompression chamber to record pressure and activity changes in the colon when desirable. The results indicate that when 300 cc. of room air are injected at ground level there occurs the expected progressive increase in collectable flatus volume as one goes to altitudes up to 25,000 feet in our experiments. The composition of a series of collected flatus samples on 3 different subjects showed that the percentage of CO<sub>2</sub> changes from 9.00% at ground level to 21.18% at 25,000 feet, while the O<sub>2</sub> concentration of flatus decreases under the same conditions from 14.14% to 9.95%. The time allowed for these changes to occur is no more than 45 minutes. The mechanism responsible for this observed change is still not established. However, the ingestion of Sulfathalidine to minimize the fermentation factor had no effect on the percentage of CO<sub>2</sub> and O<sub>2</sub> composition of flatus collected at altitude.

*Method for artificially respiring animals at high pressure.* S. N. STEIN,\* R. R. SONNENSCHNEN AND P. L. PEROT, JR.\* Dept. of Psychiatry, Illinois Neuropsychiatric Institute, Univ. of Illinois College of Medicine, Chicago.

To study the effects of high-oxygen pressures on the central nervous system, a technique for artificially respiring curarized animals is described. Oxygen is administered from a cylinder to the animal via tracheal cannula, and intake and exhaust lines are regulated by

solenoid valves alternately activated by a vacuum tube relay circuit. The relay circuit is controlled by the rise and fall of a water column, in turn activated by displacement of the fluid in a non-distensible bag surrounding the chest. The animal is placed in a chamber into which nitrogen is admitted to increase the ambient pressure. The pressure of the oxygen administered to the animal is automatically equilibrated to the pressure of the surrounding nitrogen. Electro-servo-mechanism control permits adjustment of respiratory excursion while the experimental animal is sealed in the chamber. EEG, EKG, polarographic oxygen tension, pH, and servo-mechanism leads are brought through the walls of the chamber in pressure sealed fittings. The technique is suitable for studying the effects of other gases beside oxygen at atmospheric pressure or higher.

*Effects of certain chemical agents on survival of the mouse to explosive decompression.* F. W. STEMLER,\* J. E. WIEBERS\* AND W. A. HIESTAND. Lab. of Animal Physiology, Purdue Univ., Lafayette, Ind.

The effects of 17 chemical compounds on survival to explosive decompression at a barometric pressure of 105 mm. Hg (equivalent to 3% oxygen at sea level pressure) were measured with mice of the Swiss and Hygienic strains. This pressure in the mouse is equivalent to complete anoxic anoxia. Substances increasing survival time are the following: (Percentage increase above normal is indicated by %) propanol 201%, ethanol 177%, methanol 85%, epinephrine 89%, posterior pituitary extract 67%, iodoacetic acid 52%, morphine 27%, glucose 29%, glucose plus epinephrine 24%, desoxycorticosterone acetate 9% and chloralose 5%. Substances decreasing survival time are the following: (Percentage decrease below normal is indicated by %) ephedrine 33%, prostigmine 33%, insulin 24%, lobeline 20%, ergotamine 8%.

*Chemical effects on the low-frequency pattern of squid nerve.* PETER A. STEWART (introduced by OTTO H. SCHMITT). Biophysics Group, Univ. of Minnesota, Minneapolis.

To determine the effects of ions on the membrane conductance and susceptance of the squid giant axon, the progressive changes in these characteristics during normal deterioration must be known. These time changes have been determined by the complex attenuation method. It is found that in a nerve bathed frequently with sea water, the conductance, susceptance, and threshold remain almost constant for several hours, the conductance subsequently rising to very high values while the susceptance decreases gradually. Excitability is lost when the conductance reaches about 4 milliohms/cm.<sup>2</sup> (measured at 100 cps). Not washing the nerve causes a marked shortening or even elimination of the steady state part of this pattern. However, the general pattern seems to be completely irreversible

and inevitable. Changes in the membrane characteristics produced by experimental agents are superimposed on it. The effects on the membrane conductance and susceptance of potassium, calcium and magnesium ions have been explored. It is found that the conductance is very sensitive to changes in concentration of these ions, while the 1000-cycle susceptance is relatively insensitive. Potassium and magnesium act somewhat similarly, an excess of either raising the conductance reversibly, a deficiency lowering it, although decreasing the potassium ion concentration has only a slight effect. An excess of calcium lowers the conductance reversibly, while a decrease in the calcium concentration raises it.

*Hyperglycemic response in pups to anoxic anoxia.* J. CLIFFORD STICKNEY, DAVID W. NORTHUP AND EDWARD J. VAN LIERE. Dept. of Physiology, School of Medicine, West Virginia Univ., Morgantown.

Twenty-two healthy pups (3.08 kg. av. wt.) which were fasted approximately 24 hours and which had never been exposed to anoxia were exposed without anesthesia in a decompression chamber to a simulated altitude of 28,000 ft. (254 mm. Hg barometric pressure) for 15 minutes. The fasting blood sample was drawn from the saphenous vein immediately before ascent, which was made in about 2½ minutes. The descent, at the end, took about 20 seconds, and the second sample was drawn within ¼ minutes on the average. Blood sugar was determined by the Folin-Wu method. The average control blood sugar of the pups was 96.1 (range: 76-118) and the average value after anoxia was 117.5 (range: 77-173) mg/100 ml. The difference, 21.4, is statistically significant at less than the 0.1% level. A similarly treated group of 14 adult dogs studied at a different time and reported previously had a considerably greater hyperglycemic response. The anoxic blood sugar value in the dog was 145 representing a rise of 41 mg/100 ml. These two values are significantly greater than those in the pup at less than the 0.1 and at the 1.5% levels, respectively. No adequate explanation for the lower hyperglycemic response of the pup can be made at this time.

*Alterations in connective tissue mast cell induced by bacterial pyrogens.* EDWARD G. STUART (introduced by W. F. WINDLE). Dept. of Anatomy, Univ. of Pennsylvania, Philadelphia.

The connective tissue mast cells in the abdominal skin of the mouse were studied by the technique of Zahl and Nowak. In normal animals they are especially numerous along capillaries and small venules. Elsewhere in the connective tissue they occur in scattered groups. Their granules are typically concentrated about a central nucleus so that the cells have a round or oval appearance. A few have indefinite cell boundaries due to dispersion of the granules. Highly purified bacterial pyrogenic extracts (Baxter Pyromen)

were administered intraperitoneally and orally. Dosages varied from 507/kg. to 57/kg. the latter dosage given constituting an arbitrary standard for purposes of comparison of results under varied conditions. Skin biopsies were taken at intervals varying from 15 minutes to 30 days after injections. After Pyromen administration, the granules of many mast cells dispersed and disappeared. In the immediate vicinity of small vessels the dispersion began between 5 and 15 minutes after administration depending on concentration of dosage. The groups of mast cells lying some distance from blood vessels were affected less rapidly and in a somewhat different manner. The shape of the mast cells changed; they became less discretely ovoid, the granules spread out widely away from the cell center and many of the pericapillary cells became spindle shaped. The response to Pyromen followed a curve of activity which reached its peak 7 to 10 days after a single 57/kg. dose. Numerically, morphologically and spatially it followed a definite pattern dependent on the dosage, elapsed time and strain of experimental mice employed. (Aided by a grant from Baxter Laboratories Inc., Morton Grove, Ill.)

*Changes in renal blood flow, extraction of inulin and creatinine, and glomerular filtration rate following acute alterations of renal artery perfusion pressure.*  
R. S. STUDY AND R. E. SHIPLEY. Lilly Lab. for Clinical Research, Indianapolis General Hospital, Indianapolis, Ind.

In anesthetized dogs the perfusion pressure to one kidney was altered for periods of 10 minutes at levels ranging from 20 to over 320 mm. Hg and simultaneous measurements were made of renal blood flow (rotameter), extraction per cent inulin and creatinine, and glomerular filtration rate. From perfusion pressures of approximately 20 mm. Hg, renal blood flow, extraction per cent inulin and glomerular filtration rate increased rapidly up to approximately 80 mm. above which all 3 functions remained relatively constant until 180 mm. Hg was reached. Above 180 mm. renal blood flow increased rapidly, extraction per cent of inulin decreased and the resulting glomerular filtration rate remained relatively unchanged. The contours of extraction per cent creatinine plots were essentially the same as those of inulin, but the absolute extraction per cent of creatinine was generally less than that of inulin in the normal pressure-flow range (80-180), and at low renal blood flows extraction per cent of creatinine was almost always negative. Negative extraction per cent creatinine values (to minus 38 per cent) were found below 60 mm. Hg while perfusion pressure was being either raised or lowered. The calculated quantity of creatinine present in the renal interstitial fluid was insufficient to account for the negative values. It would appear that creatinine (or other chromogenic substance) was added to renal vein blood by the kidney. The amount of creatinine-like substance contributed per minute to renal vein

blood was relatively constant regardless of changes in renal blood flow or renal artery perfusion pressure.

*Oxygen utilization of various functional areas of dog cerebral cortex.* D. L. STUMPF,\* R. J. CAFFEY,\* AND H. C. STRUCK. Dept. of Physiology and Pharmacology, Creighton Univ. School of Medicine, Omaha, Nebr.

Studies of effects of drugs or other experimental procedures on respiration of cerebral cortex have been made in the past with the assumption that all areas of the cortex respire at the same rate under normal conditions. To determine the validity of this assumption, oxygen utilization of various functional areas of dog cerebral cortex was determined by Warburg technique. Dogs were chosen because the brain is large enough to give suitable specimens for this method of study and the cortex has been mapped with sufficient accuracy to enable separation of at least the major functional areas. Animals were killed without anesthesia, the brains removed, and slices prepared in a moist-cold box. The slices were weighed and suspended in Krebs-Ringer phosphate, pH 7.4, with dextrose substrate. Oxygen was the gas phase. Mean 3 hour  $QO_2$ 's for slices obtained from 17 dogs is shown in the table.

Area	No. of Slices	Mean $QO_2$ (3 hours) mm <sup>3</sup> /100 mg. wet tissue
Temporal (2)	44	454 ± 60 (S.D.)
Sensory	40	435 ± 57
Motor	65	474 ± 54
Temporal (1)	42	400 ± 52
Optic	31	398 ± 49
Olfactory	36	347 ± 47
Auditory	33	330 ± 48

Statistical analysis of these results indicates that differences between means of 21 or more are significant. It is evident that the cerebral cortex is not uniform with respect to its rate of metabolism as measured by oxygen uptake.

*Point of irreversible cerebral insult during the process of death.* H. G. SWANN. Univ. of Texas Med. Branch, Galveston.

During the process of death the organism experiences anoxia for so long that it eventually comes to a point where an irreversible cerebral insult is done. This point was quantitated in dogs for 4 different types of anoxic death. The animals' breathing and circulation were restored when necessary, during the death process, by artificial respiration and extrathoracic cardiac massage. All animals were carefully nursed and artificially fed after the experience. The animals were designated as 'resuscitated' when complete recovery occurred; they were designated as 'vegetalized' when such an overwhelming cerebral insult was done that they lived with-

out cerebral function for only a few days. The points of resuscitation and vegetalization were found to be as follows: for the fulminating anoxia of breathing pure  $N_2$ ,  $5\frac{1}{2}$  minutes and  $6\frac{1}{2}$  minutes, respectively; for obstructive asphyxia, 2 minutes after circulatory failure and 3 minutes afterwards respectively, the two points being independent, within limits, of elapsed time; for the acute anoxia of breathing 2.43%  $O_2$  in  $N_2$ , just at circulatory failure and one minute after circulatory failure respectively, the two points being independent of elapsed time. In carbon monoxide poisoning (1% CO in air), the irreversible cerebral insult was done in two-thirds of the dogs definitely before circulatory and respiratory failure.

*On Pflüger's tetanus in frog motor nerve fibers.* P. O.

THERMAN. Hall-Mercer Hospital, Pennsylvania Hospital Division, Philadelphia.

The repetitive discharge of nerve impulses in response to cathodal stimulation, known as Pflüger's tetanus, has been studied in nerve-muscle preparations of bullfrogs. The common sciatic nerve was stimulated with 10  $\mu$ amp. currents of 10 to 500 msec. duration and the isotonic contractions of both gastrocnemius and tibial muscles were recorded separately. Since a repetitive discharge will give a tetanic contraction greater in height than a single maximal twitch the occurrence of repetitiveness in motor nerves can be studied under various conditions. Repetitive discharges are easily obtained with weak cathodal currents in motor nerves that have been kept in 5%  $CO_2$  and 95%  $O_2$  but not if the nerves have been kept in pure oxygen for more than 30 minutes. A marked facilitation of the repetitive response occurs in carbon-dioxide-treated nerves whenever the  $CO_2$  tension is being decreased, whereas these responses are abolished during periods of increasing  $CO_2$  tension. No repetitive response is obtained in pure nitrogen, even during decreasing  $CO_2$  tensions, provided the  $CO_2$  had been added in the absence of oxygen. Motor nerves treated with 0.015 M tetraethylammonium chloride respond with a profound Pflüger's tetanus in the presence of carbon dioxide, but only with a single twitch in pure oxygen. These effects are more pronounced in the flexor group. Thus, Pflüger's tetanus is intimately connected with an oxidative reaction of the motor nerve fibers to carbon dioxide, specifically affected by depolarization causing repetitive discharges of impulses.

*Effect of electrical stimulation on muscular atrophy resulting from acute inanition.* J. D. THOMSON\*, A.

BRODISH\* AND H. M. HINES\*. Dept. of Physiology, State Univ. of Iowa, Iowa City.

Studies were carried out concerning the effect of electrical stimulation of the gastrocnemius muscle of rats while undergoing atrophy during acute inanition and while recovering from the effects of a fast in which there occurred a body weight loss of 30%. The stimulus

consisted of direct stimulation for one minute, twice a day, with repetitive discharges from a Harvard Inductorium, with the secondary coil set 4 cm. from the primary. The muscles were kept fully stretched during the treatment. It was found that the electrical stimulation retarded loss of muscle weight and creatine content when applied during periods of acute inanition. This treatment tended to retard loss of total muscle strength, but not strength per unit of fasting muscle. When electrotherapy was applied to the muscle during a refeeding recovery period of 15 days duration, it caused a small increase in weight but not in strength or creatine concentration of the treated muscle. Thus, electrotherapy is effective in retarding the loss of weight from skeletal muscle during acute inanition but does not prevent the loss of strength per unit mass of tissue. This is in accord with previously noted effects of electrical stimulation on denervated muscle and on spastic muscle.

*Effect of pituitary and adrenocortical hormones on neuromuscular function.* CLARA TORDA AND HAROLD G.

WOLFF. New York Hospital, the Kingsbridge Hospital (V.A.), and the Depts. of Medicine (Neurology) and Psychiatry, Cornell Univ. Med. College, New York City.

After hypophysectomy nerve function is impaired as evidenced by the decrease of action potential during repetitive stimulation and the decrease of ability to synthesize acetylcholine. Muscle function on direct stimulation, however, is not significantly impaired. In the following an attempt was made to ascertain the effect of some hormones secreted by the pituitary gland, adrenalectomy and cortisone on the function of nerve and muscle. Muscle function was tested by myography, nerve function by action potential measurements, and acetylcholine synthesis by a modified method of Quastel, Tennenbaum and Wheatley. Adrenalectomy modified only slightly the behavior of the nerve action potential and the ability to synthesize acetylcholine, but induced a marked fatigability of the muscle. This dysfunction was partially reversible by cortisone. Both thyroxine and the thyrotrophic hormone of the pituitary gland induced in both unoperated and hypophysectomized animals a decrease of the function of nerve and muscle and a decrease in the ability to synthesize acetylcholine. Adrenocorticotrophic hormone increased the ability of unoperated animals to synthesize acetylcholine and restored the function of nerve and the ability to synthesize acetylcholine to normal in hypophysectomized animals. Cortisone restored the nerve function of hypophysectomized animals only partially. Thus, the pituitary gland is an important factor in the maintenance of neuromuscular function at an optimum level. Seemingly, the dysfunction of nerve induced by hypophysectomy is a result of a dys-equilibrium between the hormones secreted by the adrenal gland rather than the result of the absence of the gland. The effect

of pituitary gland in maintaining optimal neuromuscular function is mediated by ACTH.

*Protein complexes and the impermeability of the blood-brain barrier to dyes.* R. D. TSCHIRGT (introduced by R. W. GERARD). Univ. of Chicago, Chicago, Ill.

Intravenous Trypan Blue (T.B.) in initial plasma concentrations up to 0.2% left rat brains unstained, while deeply staining other organs after 45 minutes. When placed directly on the cortex for one hour, 0.2% T.B. in plasma left brain colorless (surrounding dura, fascia and muscles deeply stained), while 0.2% T.B. in Tyrode solution stained brain intensely, to a depth proportional to duration. Similar results were obtained with fluorescein, in concentrations up to 0.04%. Addition of bovine plasma albumin to a Tyrode solution of fluorescein or T.B. inhibited staining of brain (cortical application) much more than of surrounding tissues. Basic dyes (e.g. pyronin) in plasma (topically or i.v.) stain the brain in extremely dilute concentrations. It is known that basic dyes are less strongly and regularly bound by plasma protein than are acid dyes. Using Bennhold's gelatin diffusion technique, it was shown that the staining of brain (under the above conditions) is directly related to the amount of dye not bound to protein and to its diffusibility in gelatin. Gelatin, separated from various protein-dye solutions by a protein impermeable membrane, stained similarly to rat brain following topical application of these same solutions. Intravenous T.B. (0.11%) penetrates to, but stops sharply at, the perivascular pia-glia membrane of the brain *in situ*. From comparable liver vessels, the dye diffuses outward into the parenchyma. It is suggested that the impermeability of the pia-glia membrane to protein, or its complexes with a variety of drug and other molecules, accounts for much of the blocking action of the blood-brain barrier.

*Ultra-centrifugal fractionation of hemolytic and anti-hemolytic agents in different adult and fetal guinea pig tissues.* DAVID B. TYLER. Dept. of Embryology, Carnegie Institution of Washington, Baltimore, Md.

Simple saline extracts of fetal guinea pig liver possess a heat sensitive agent capable of rapidly hemolyzing erythrocytes from the same animal, from those of the mother or those from a different species. Similarly prepared maternal liver extracts are inactive in this respect (*Proc. Soc. Exper. Biol. & Med.* 72: 491, 1949). This report is concerned with 1) the nature of this hemolytic agent, 2) its relation to the general problem of blood destruction, 3) the reason for this unusual fetal-maternal activity difference, particularly the lack of activity in maternal preparations and 4) the presentation of substantial evidence that differences in rate of certain enzymatic activities, as found between fetus and adult are not necessarily due to differences in enzyme concentration but may be accountable on the

basis of the effective activity or concentration of inhibitory regulating mechanisms. Experiments demonstrate the presence in the liver of an inhibitor as well as the hemolytic agent. In the adult these substances exist in such proportions that the activity of the enzyme (or lytic agent) is inhibited. Separation can be made by ultra-centrifugation with the result that the inhibitor is found to be associated mainly with the fraction sedimenting at  $25,000 \times g$  (mitochondria) while the hemolytic agent remains in the supernate after  $100,000 \times g$  for one hour. Such supernates of adult livers possess high hemolytic activity equal to that found for fetal preparations. Recombining the various adult fractions (the supernate with the separated mitochondria and microsomes) restores the inactive state. Recombining adult supernates with various amounts of fetal mitochondria or microsome fractions demonstrates that these latter fractions, in contrast to those of the adult, possess very little anti-hemolytic (inhibitor) activity.

*Oximeter earpiece and recording assembly for measurement of systemic blood pressure and arterial oxygen saturation in man* (Demonstration). JOHN W. UZMANN\* AND EARL H. WOOD. Mayo Foundation, Univ. of Minnesota, and Mayo Clinic, Rochester, Minn.

A two-channel brush direct-writing oscillograph and suitable amplifiers have been adapted to the objective measurement of systemic blood pressure in man, by use of the method described by Wood and others (*Federation Proc.*, 9: 139, 1950) and a Waters-Conley oximeter earpiece of the absolute-reading type. The red cell of the earpiece is used to pick up the ear-opacity pulse which is amplified and recorded on one channel of the ink-writing oscillograph. The earpiece capsule pressure required to obliterate the pulse (systolic) and at which the pulse is at maximal amplitude (diastolic pressure) is measured with a strain-gage amplifier assembly recording on the second channel of the oscillograph. A multipole switch is used to connect the earpiece at will to a single-scale, absolute-reading ear oximeter (*Am. J. Physiol.* 159: 597, 1949). Therefore, it is possible to obtain from the same earpiece rapidly successive measurements of either the arterial pressure or oxygen saturation. The accuracy of the device has been studied in a series of 10 normal subjects by comparing the results of simultaneous measurements of systemic pressure at the ear and the arterial pressure obtained by direct puncture of the radial artery and by the usual indirect auscultatory method. A comparison of manometric values with oximeter readings of saturation obtained during withdrawal of the arterial samples during the breathing of air, oxygen and low oxygen mixtures has been made in the same subjects. The accuracy of the earpiece determinations of both arterial pressure and oxygen saturation was similar to

that obtained previously with the use of photokymographic recording without amplification (*Proc. Staff Meet., Mayo Clin.* 25: 384, 1950).

*Apparatus and technique for study of explosive decompression.* EDWIN G. VAIL, JOHN P. KEMPH, AND FRED A. HITCHCOCK. Lab. of Aviation Physiology and Medicine, Ohio State Univ., Columbus.

The Laboratory of Aviation Physiology was open for inspection. Apparatus used in the study of explosive decompression was demonstrated. This included a large standard, 20 man Air Force decompression chamber, a Tinney stratosphere chamber for temperature-low pressure studies, and an animal chamber designed for use with a portable x-ray machine. Technique for performing explosive decompression was explained. Methods used in studying various physiological responses to explosive decompression such as blood pressure, ECG, and EEG were shown. A thermistor was demonstrated as a rapid temperature measuring instrument.

*X-ray studies of lungs and G.I. tract following explosive decompression.* EDWIN G. VAIL, DONALD ROSENBAUM AND FRED A. HITCHCOCK. Lab. of Aviation Physiology, Ohio State Univ., Columbus.

In connection with investigations on the physiological effects of explosive decompression, it was thought that many of the problems which have arisen could be clarified by x-ray studies of organ systems. Forty dogs were explosively decompressed to 30 mm. Hg, using various radiological techniques for visualization of organs. Lipiodol was used in the studies on the pulmonary tract, barium for the gastrointestinal tract, and prodiol for the gall bladder. The lung studies showed massive 'vapothorax' 30 seconds after explosive decompression in all animals. This lung collapse was probably due to the vaporization of interpleural fluids. The shape of the thoracic cage changed from a triangular shape before E.D. to a more rounded shape after decompression, which probably reflects the changes in the interpleural pressure. On recompression, the cross-sectional area of the thoracic cage decreased. The gall bladder showed no changes except in position, moving from a central to a more lateral position, probably due to the massive dilatation of the stomach. Since the stomach was greatly distended, barium was almost always regurgitated, with some aspiration into the trachea and bronchi on recompression. The esophagus was greatly dilated, and could be seen to contain many gas bubbles in the barium film. The small and large intestines were greatly distended by gas. Subcutaneous and intermuscular emphysema were noted in all animals by 30 seconds after the decompression. 'Vaparthrosis' was observed in many joints. Intracardiac gas may be visualized with x-ray 30 seconds after explosive decompression. (This research was supported

under contract with the AeroMedical Lab., Wright-Patterson Air Force Base, Dayton, Ohio.)

*Effect of anoxic anoxia on motility of the small intestine in pups.* EDWARD J. VAN LIERE, J. CLIFFORD STICKNEY AND DAVID W. NORTHPUP. Dept. of Physiology, West Virginia Univ. School of Medicine, Morgantown.

It has been shown (*Am. J. Physiol.* 140: 119, 1950) that even severe degrees of anoxic anoxia do not affect the propulsive motility of the small intestine of adult dogs, in contrast to its effect on mice and rats. It seemed worthwhile to study the problem on pups. Twelve matched pairs of pups which had been fasted 24 hours were used, one to serve as control and the other as the experimental animal. About 25 cc. of a mixture of 10% charcoal suspension in 10% gum acacia in water was given by stomach tube. Three minutes later the experimental animal was placed in a low-pressure chamber and subjected to an atmospheric pressure of 246 mm. Hg, simulating an altitude of 28,000 feet. At the end of 30 minutes the animal was removed from the low-pressure and killed with ether. The small intestines were removed, slit open and the distance the charcoal had traversed the intestine measured. The control animals were similarly treated. In the control animals the charcoal traveled an average of 75.4% of the length of the intestine, whereas the experimental animals showed an average of only 51.1%. The difference was statistically significant at the 1% level. It was concluded that unlike the adult dog, the propulsive motility of the small intestine of the pup is distinctly affected by severe degrees of anoxic anoxia.

*Inhibition of gastric secretion and ulceration by parasympatholytic drugs.* FRANK E. VISSCHER AND ADRIAN P. TAZELAAR. Dept. of Pharmacology and Endocrinology, Upjohn Co., Kalamazoo, Mich.

We have determined the anti-secretory activity in pyloric ligation rats of a number of parasympatholytic compounds. It has been observed that the ED<sub>50</sub> (dose to block by 50% the gastric secretion of pyloric ligation rats during the first 2 hours after i.v. injection of the drug) for certain tertiary amines ranges between 0.5 and 5.0 mg/kg. The compounds are of the Cyclopyrazate type: di- or tri-substituted (alkyl, aryl, hydroxyl) acetates or unsubstituted or 2-methyl-substituted pyrrolidyl ethanolols. The corresponding quaternary compounds (etho- or metho-chlorides or bromides) have an enhanced activity (three to tenfold in four pairs) with ED<sub>50</sub>'s between 0.2 and 1.0. The quaternary derivatives have been shown to prevent starvation ulcers in rats when given by daily subcutaneous injection during the period of starvation. The dose required is 2 mg/kg/day in divided dosage in aqueous solution or as a single injection in oil. Banthine is likewise effective.

*Effect of excess salt ingestion upon blood pressure of normal dogs.* E. B. WALDMANN\* AND C. M. WILHELMJ. Dept. of Physiology and Pharmacology, Creighton Univ. School of Medicine, Omaha, Nebr.

Three standardized dogs were given excess salt (2.5 gm/kg.) in the diet for 205, 56 and 154 days. The first effect was a marked increase in the daily fluctuations and a small but significant elevation of systolic pressure. Diastolic pressure was elevated in only one dog. Finally the fluctuations diminished and the systolic pressure returned to normal in 2 dogs, but remained significantly elevated in one (*Salt Basal Period*). The salt stress was now increased by giving 2% saline as drinking water for 49, 49 and 35 additional days. One dog showed a significant elevation of systolic pressure above the salt basal level, the other 2 did not. These experiments show a progressively increasing adaptation to excess salt. When the salt basal period was established the salt was continued and the dogs were subjected to a second stress, a slowly revolving cage for 3 to 18 hours daily. The first effect was a prolonged and marked elevation of systolic and diastolic pressure, but as the second stress was continued the effect became less and finally nil. Thus adaptation to a second stress occurred while the first stress continued. The total period on excess salt was 254, 105 and 189 days. When excess salt was suddenly discontinued the systolic and diastolic pressure underwent a marked and highly significant drop below the normal control level. This new low level persisted for at least 3 weeks. This post stress drop is possibly related to pituitary and adrenal cortical function.

*A simple method for determination of pancreatic amylase.*

JEROME M. WALDRON (introduced by IRVING H. WAGMAN). Dept. of Physiology, Jefferson Med. College, Philadelphia, Pa.

The hydrolysis of a starch suspension by pancreatic amylase is a monomolecular reaction. Therefore, the activity of pancreatic amylase may be expressed as the velocity constant of this reaction if a stable starch suspension is used. A starch suspension which is stable indefinitely, even withstanding freezing and thawing, is now available. This standard starch substrate with sodium chloride and phosphate buffer to give optimal amylolytic activity is used as the combined substrate for digestion by diluted pancreatic juice. The rate of digestion is determined by measuring photoelectrically the decrease in turbidity in time of the combined substrate. Amylolytic activity is expressed as the velocity constant of this monomolecular reaction. Evidence is presented that under the conditions of the procedure the reaction behaves as a monomolecular one and that the described procedure gives reproducible results. The described procedure has the advantage that a comparison can be made with other enzymes because the activity of pepsin and trypsin can be measured

in the same manner. In addition the expression of amylolytic activity as the velocity constant of the reaction has physico-chemical significance.

*Depression of gastric secretion with pyrogens and antipyretics without fever.* L. WALKER, WM. H. OLSON AND H. NECHESLES. Dept. of Gastro-Intestinal Research, Med. Research Institute of Michael Reese Hospital, Chicago, Ill.

It has been frequently confirmed that a rise in body temperature may cause a partial or complete inhibition of hydrochloric acid secretion of the stomach. Injection of pyrogens can produce high temperature plus inhibition of gastric secretion. We have reported that pyrogens given in doses that did not produce fever were able to depress gastric secretion, but this has been contested. Our present results on dogs with total gastric pouches and histamine stimulation show that pyrogens given in subpyretic doses may have some inhibiting effect. This effect is not constant, most animals showing no inhibition of gastric secretion with low doses of pyrogens. We have obtained consistent inhibition of gastric secretion with large doses of pyrogens that produce a rise in body temperature. By use of antipyretics the temperature rise was abolished. However, the marked inhibition of gastric secretion still occurred. With a dose of 50 gammas of a purified bacterial pyrogen (Pyromen) plus 10 grains of aspirin by mouth no rise in rectal temperature occurred. There was an average 60% reduction in the total volume of gastric juice and a 72% reduction in total mEq. of free hydrochloric acid. The latent period for the effect of the pyrogen was from 30 to 60 minutes. (This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.)

*Relation of quick stretch and tetanus in skeletal muscle.*

SHEPPARD M. WALKER. Dept. of Physiology, Univ. of Louisville School of Medicine, Louisville, Ky.

The *in situ* triceps surae of the rat was stimulated through the cut sciatic nerve and stretch was applied after the plateau of isometric tetanus was attained. Stretch was completed in 5 to 10 msec. by displacing the fixed end of the muscle with a strong spring. At the muscle length required for development of maximal tetanic tension tetanized muscle is less extensible than the resting muscle at the same length. On the other hand, the tetanized muscle is more extensible than the muscle resting at a passive tension equal to tetanic tension. The decrease of extensibility in tetanized muscle is proportional to the increase of initial length of the muscle. Quick stretch brings about an increase of tension which temporarily exceeds the tension attainable by isometric recording, at the length attained by applying the quick stretch. The tension usually declines after the application of quick stretch during tetanus. When length-tension equilibrium is reached after stretch the tetanic tension is about equal to ordinary

isometric tetanus at the same muscle length. The tension usually rises after quick stretch if tetanus is incomplete. Action potential records indicate that quick stretch does not alter the number of muscle fibers responding during tetanus. It is concluded that 1) interpretations of changes in extensibility of tetanized muscle depend on the length and tension of the resting muscle used for comparison, and 2) the tension in excess of isometric tetanic tension, observed when quick stretch is applied during tetanus, is passive.

*Exchange of potassium in plasma, liver and muscle.*

W. GORDON WALKER\* AND WALTER S. WILDE. Physiology Dept., Tulane Univ., New Orleans, La., and Biology Division, Oak Ridge Natl. Lab., Oak Ridge, Tenn.

As previously reported (*Am. J. Physiol.* 159: 594, 1949), disappearance of intravenous  $^{42}\text{K}$  in rabbits is extremely rapid, 90% of the injected tracer disappearing within one minute. Multiple early sampling yields a more precise description of plasma concentration. For  $t$  up to 50 minutes the best fit is:  $P^* = 26.1 e^{-2.34t} + 0.94 e^{-0.11t} + 0.14$ ; the coefficients are cts/ml/cts injected/gm. body wt. The coefficient 26.1 corresponds to a plasma volume of 4% and thus suggests early circulatory mixing. Experimental points are in good agreement with the above equation at all times after 20 seconds. Since the extravascular mass of K is so large that early backflow of tracer may be ignored the 2.34 represents the turnover rate of the plasma K mass. This seems circulation limited since it approximates cardiac output. Variation of  $[\text{K}]$  in the injected solution between 3.5 and 1340 mM/l. affects neither the slope nor intercept and thus argues against net transfers of plasma K for homeostasis. That the initial rapid process is not solely an exchange into visceral organs (liver) may be inferred from curves for eviscerated animals. Portal injection substantiates this. The second phase of the plasma curve is an interaction of several events. At 50 minutes plasma specific activity ( $\text{K}^*/\text{K}$ ) actually rises. This peak of variable height could not occur by reversible exchange. It is obliterated by portal injection and by evisceration. Subsequent decline is exponential, the best fit being  $P^* = 0.6 e^{-0.018t} + 0.07$ . This phase probably corresponds to the plasma curve described for the dog by Levitt and Gaudino, and represents a plasma-to-muscle exchange after viscera have equilibrated, rather than one exchange rate for all tissues, as they suggested. Analyses at 2 minutes reveal that the viscera, representing 10% of the body weight and body K, contain 55% of the  $^{42}\text{K}$ . The kidney is apparently equilibrated; muscle contains little more than that present in Cl-space. Muscle exchanges so slowly that the late plasma and muscle curves represent a two-compartment system. The turnover rate is 0.002/minute. From 2 to 24 hours  $\text{K}^*/\text{K}$  remains steady at a value suggesting 80%

exchange. After portal injection liver  $\text{K}^*/\text{K}$  equals plasma 10 to 35 minutes sooner, and no subsequent rise is noted.

*Chronic hypervagotonia.* R. N. WATMAN\* AND E. S. NASSET. Dept. of Physiology and Vital Economics, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The lack of a satisfactory chronic animal preparation accounts for the paucity of information regarding the effects on the alimentary tract of prolonged vagal stimulation. Acute experiments, using electrical stimulation, are usually done under anesthesia and at best can be continued for only a few hours. In the present study the right vagus and left phrenic nerves were divided above the diaphragm. The proximal end of the divided phrenic was anastomosed to the distal end of the divided vagus. The other vagus was sectioned and one cm. excised. Changes were observed, post-operatively in gastric secretion and motility which may be attributed to regeneration of the phrenic into the vagal pathways.

*Simplification of method of Greenberg and Lester for determination of ketone bodies.* HARRIET R. WEINSTEIN\* AND PIERO P. FOÀ. Dept. of Physiology and Pharmacology, Chicago Med. School, Chicago, Ill.

Greenberg and Lester (*J. Biol. Chem.* 154: 177, 1944; 174: 903, 1948) described a micro-method for the determination of ketone bodies in blood and urine based on a color reaction between acetone and 2,4-dinitrophenylhydrazine. In this method the ketones are oxidized to acetone in a micro-refluxing apparatus requiring constant attention. Furthermore, unless one possesses several apparatuses, the simultaneous analysis of numerous samples is impossible. It was found that the oxidation of ketones can be carried out satisfactorily in a screw-capped test tube. Blood and urine filtrates are prepared as described by Greenberg and Lester. Three cc. of the supernatant fluid are delivered into a screw-capped test tube (120 by 20 mm.) with a bakelite cap 17 mm. deep and lined with acid-resistant plastic; 0.6 cc. of acid dichromate are added, the tube closed tightly and immersed in hot water to check for leaks (air bubbles) around the cap. The tube is then boiled in water for 10 minutes, cooled thoroughly and opened to allow the introduction of 0.5 cc. of 10% dichromate. The tube is then again tightly closed, checked for leaks and boiled for an additional 10 minutes. After cooling the procedure is continued as described in the original method, except that Hennessy funnels are used instead of 10-cc. stoppered graduates to make the transfer of the solution to the colorimeter tubes easier. This procedure allows the simultaneous duplicate analysis of many samples of blood and urine. The results are comparable to those obtained with the original method.



*Physico-chemical properties of plasma proenzymes.* ANDRE M. WEITZENHOFER\* AND EARL B. GERHEIM. Division of Basic Sciences, Univ. of Detroit and Wayne Univ. College of Medicine, Detroit, Mich.

A human proenzyme preparation has been obtained which under optimum conditions has 70% purity as determined electrophoretically (Klett Apparatus). In general, electrophoresis produces four components below pH 6.5 and above pH 7.0. These components have been found to have electrophoretic isoelectric points around pH 4.4, 5.1, 6.6, and 7.5. All but one appear to be identical with known plasma components. The major component (50-60%) appears to be a gamma globulin. The carbohydrate content (Orcinol test) appears to be around 6.80%, and the tyrosine (Folin-Ciocalteu) seems to be about 3.78%. The acetone-ether dried product is quite stable over a period of several months. Heat stability studies at 37°C., 45°C., 56°C. and 70°C. indicate the proenzyme to be more stable than the active enzyme.

*Cerebellar projections to the upper brain stem including the basal ganglia.* JAMES A. WHITESIDE AND RAY S. SNIDER. Univ. of Alabama Med. College, Birmingham, Ala. and Northwestern Univ. Med. School, Chicago, Ill.

An oscilloscopic study of the responsive areas of the upper brain stem to stimulation of the cerebellar cortex was undertaken because of the increasing significance of the cerebellar role on the extrapyramidal system. The brain stem was explored with bipolar electrodes, oriented stereotactically, during electrical stimulation of various functional areas of the cerebellum. Responses with less than 0.5 msec. latencies, consequently without synapse were recorded: a) at midbrain levels, in decussation of brachium conjunctivum, adjoining tegmentum and central gray substance, and in the pretectum, b) at thalamic levels, in subthalamus and ventral thalamic nuclei (esp. ventro-lateralis and ventro-postero-medial) adjacent to the intramedullary lamina. Responses of longer latency but shorter than 3 msec. were found in substantia nigra, medial geniculate nucleus, superior colliculus, optic tract, cerebral peduncle, and globus pallidus. Responses with latencies between 4 and 8 msec. were found in tegmentum, hypothalamus, substantia nigra, globus pallidus, medial geniculate nucleus, internal capsule, centralis lateralis nucleus, and centrum medianum nucleus. Occasional responses were observed in the hippocampus. Latencies of longer than 10 msec., consequently denoting multi-synaptic complexity, were recorded from putamen and internal capsule. None of the responses described above were observed in the lateral geniculate nucleus, caudate nucleus, and anterior thalamic nuclei. Electrical stimuli were usually biphasic single pulses of 0.5 msec. duration. Records from 26 cats injected with dihydro-B-erythroidine hydrobromide, D-tubocurarine chloride, and with sodium pentobarbital were studied. All punc-

tures were made in Horsley-Clarke axes, checked histologically, and the photographed responses arranged on enlarged cross sectional outlines of the histological material.

*Biotin in the metabolism of micro-organisms.* VIRGINIA WHITESIDE-CARLSON AND WARNER W. CARLSON. Med. College of Alabama, Birmingham.

Certain strains of *Leuconostoc* have been shown to require biotin in glucose or fructose media, but not in those containing sucrose (Carlson and Whiteside-Carlson. *Proc. Soc. Exper. Biol. & Med.* 71: 416, 1949). This is most marked in *L. dextranicum elai*, which utilizes sucrose only by way of converting the glucose half of the molecule to the metabolically-inactive polysaccharide, dextran. Fermentation of sucrose then is actually one of utilization of fructose, except that biotin is not required. It has now been found that D-thiobiotin is equivalent in activity to D-biotin, suggesting that *Leuconostoc* utilize it without prior conversion to biotin. This has been confirmed by the negative results from culturing *L. dextranicum elai* in a desthiobiotin medium, separating the cells and assaying the fermented medium and cell hydrolysate for biotin activity with *Lactobacillus arabinosus*. Since conversion of sucrose into dextran and fructose by cell-free extracts occurs in phosphate-free solutions, the requirement of the organisms for this ion for growth in sucrose and fructose media was determined; no difference in requirement could be demonstrated. The ability of washed, biotin-free cells to utilize sucrose or fructose in phosphate-bicarbonate buffer was determined in the presence and absence of added biotin, since the vitamin might function in the membrane transport of fructose. However, such cells utilized fructose with or without added biotin, and more readily than sucrose.

*Hemodynamic problems that enter into aortic coarctation.*

C. J. WIGGERS AND T. C. GUPTA.\* Dept. of Physiology, Western Reserve Univ. Med. School, Cleveland, Ohio.

Basic hemodynamic studies on experimental coarctation of the aorta just beyond the left subclavian artery have revealed that hitherto unsuspected physical and physiological factors are involved in creation of hypertension above a coarctation and in changes of pressure pulses below such a lesion. The effects are by no means explained by an increased resistance at the coarctation, as is generally believed. The communication analyzed the roles that changes in capacity and distensibility of the aortic compression chamber and increase in systolic discharge of the left ventricle play in the production of aortic hypertension, and discussed the physiological compensations in blood flow by which an adequate return to the right heart is maintained despite extreme reduction in flow through the inferior cava. The ways in which the pressure relations in the lower aorta and femoral artery are altered from the

normal were described and the relative shares that damping of the pulse wave and reduced input into the lower aorta play with different degrees of coarctation were emphasized. The conclusion was reached that all the dynamic changes found in experimental and human coarctation are adequately explained without the assumption of accessory vasoconstriction through reflex or humoral agencies.

*Effect of fasting upon blood pressure and heart rate of normal dogs.* C. M. WILHELMJ AND E. B. WALDMANN.\* Dept. of Physiology and Pharmacology, Creighton Univ. School of Medicine, Omaha, Nebr.

Blood pressure and heart rate were determined daily during 14 fasting periods on 4 standardized dogs. The nutritional level preceding each fast was rigidly controlled by giving a basal diet of either meat or cracker meal or a lusus consumption diet of meat plus cracker meal containing twice the basal requirements. Before starting these diets the animals were subjected to a prolonged fast. This preliminary fast exhausted the stores of labile carbohydrate and protein and the next or *experimental fast* started with only those stores which had been accumulated on the specific diet. During fasting the behavior of the blood pressure and heart rate could be divided into two stages: 1) *The Stage of the Decline to the Stable Fasting Values*. This was greatly influenced by the previous nutritional level. If the nutritional level had been high this stage was very prolonged (the longest 34 days) and was characterized by large daily fluctuations which slowly descended in an irregular saw tooth manner to the stable fasting values. If the previous nutritional level had been low this stage was short (1-7 days) and the daily fluctuations were small. 2) *The Stage of the Stable Fasting Values*. These values were very low, showed small daily fluctuations and remained unchanged for as long as 17 days. They were independent of the previous nutritional level. Since fasting is an alarming stimulus, the first stage is a counter shock phenomenon, the intensity of which is governed by the previous nutritional level.

*Acute changes in renal hemodynamics following partial occlusion of renal artery.* ARNOLD H. WILLIAMS AND EDWARD F. EDINGER (introduced by HENRY A. SCHROEDER). Hypertension Division, Dept. of Internal Medicine, Washington Univ. School of Medicine, St. Louis, Mo.

Relatively little is known about the acute hemodynamic effects of constriction of the renal artery. Several workers, using a thermostromuhr on either the renal artery or vein, have demonstrated a sustained secondary increase of renal blood flow occurring within a few minutes after constriction. This rise has been attributed to "dilatation of the renal vascular bed by autonomous forces within the kidney." (SMITH, H. W.

*Harvey Lectures* 35: 166, 1939-40.) As there is little or no evidence of renal vasodilatation in chronic experimental hypertension produced by a Goldblatt clamp, the relationship between the acute and chronic changes is not clear. Consequently the 'vasodilatation phenomenon' was subjected to closer scrutiny. Various combined studies of pressure, of resistance by the astatic arterial pressure gradient (WILLIAMS, A. H. AND H. A. SCHROEDER. *Am. J. Physiol.* 155: 132, 1948) and of direct blood flow were made in intact and denervated kidneys of anesthetized dogs. The effects of arterial constriction upon renal resistance were small and inconstant. Pressure distal to the clamp at first fell and then rose considerably during the subsequent 15 to 20 minutes. Similar changes in flow occurred. These changes were not prevented by renal decapsulation, by the application of phenol at the site of constriction or by procainization of the renal nerves. They were checked by complete denervation (rupture of the renal nerves and phenolization). Since the rise of pressure and flow was not accompanied by renal vasoconstriction, presumably the 'dilatation phenomenon' is due to changes in the caliber of the artery at the site of constriction.

*Hydrodynamics of mitral insufficiency.* FRANCIS WILLIAMS\* AND SIMON ROXBARD. Cardiovascular Dept., Med. Research Institute, Michael Reese Hospital, Chicago, Ill.

We have undertaken an analysis of the dynamics of mitral and aortic insufficiency in a specially designed artificial circulation model. This model simulates Starling's law of the heart in that with increased filling, greater work is accomplished in the ensuing contraction. Heart rate, isometric contraction, filling time, and atrial and systemic pressures can be varied at will with this model. Valvular insufficiencies are produced by preventing the ball valve from seating properly. Experiments on this model demonstrate several factors affecting the cardiac output when valvular insufficiencies are introduced. The reduction in cardiac output is much greater in mitral insufficiency than for an aortic regurgitation of the same degree. The findings given below refer to experiments with mitral insufficiency. As the venous pressure is increased, the degree of regurgitation is diminished. Ventricular output falls markedly with rising systemic arterial pressures. The effective arterial hindrance to flow is the difference between systemic and venous pressures. As the heart rate increases from very low values, the output increases until at fast rates the output falls apparently as a result of decreased filling time. An increase in the duration of isometric contraction markedly reduces forward flow. Increasing the viscosity of the fluid in the system reduces the degree of mitral regurgitation. The primary factors affecting the degree of mitral insufficiency, for a given size of leak, are the duration of isometric contraction, the difference between the venous and

arterial pressures, the heart rate, and the viscosity of the fluid.

*Extramedullary hemopoiesis in rabbit and cat induced by bacterial pyrogens.* WILLIAM F. WINDLE AND HARRY H. WILCOX. Dept. of Anatomy, Univ. of Pennsylvania, Philadelphia.

Pyromen (Baxter) was administered to rabbits and cats in a wide range of dosages for varying lengths of time. Histological changes in the spleen were related to the length of time of dosages and to the size of dose, the former being more important. Changes, most marked in the red pulp, involved proliferation of reticular cells and reduction of blood flow through the splenic sinuses. These changes occur in all animals receiving Pyromen for 20 days. By 30 days static blood was phagocytized by macrophages and groups of hemopoietic cells appeared in the pulp cords. Shortly thereafter islands of hemopoietic tissue, resembling blood islands of a developing embryo, appeared within the splenic sinuses. With prolonged or massive dosages of Pyromen there was such an increase in numbers of myelocytes, normoblasts and megakaryocytes that the spleens resembled bone marrow. Reaction to Pyromen was similar in rabbits and cats. However, with long period administration in rabbits there was only a slight increase in weight of the spleen, while in cats a great increase was observed. In the sinuses of the rabbit liver, hemopoiesis, which was not found in any of the control animals nor in any receiving low doses of Pyromen for less than 21 days, appeared in animals with prolonged and/or massive dosages. Extramedullary hemopoiesis was confined to the hepatic sinuses and involved production of myelocytes and megakaryocytes only. (This study was aided by a grant from Baxter Laboratories, Inc., Morton Grove, Ill.)

*Study of minimal dynamic response characteristics of manometer systems required for adequate recording of peripheral arterial pressure pulses in man.* EARL H. WOOD (with the technical assistance of ROY ENGSTRÖM). Mayo Foundation, Univ. of Minnesota, and Mayo Clinic, Rochester, Minn.

Intrabrachial arterial pressure was photographed continuously while intraradial pressure was simultaneously recorded from the same arm with the use of 5 different manometer systems of widely different dynamic response. The brachial arterial pressure served as a base for study of the effects of variation in dynamic response of the radial manometer on the recorded radial pressure. The dynamic response of the radial manometer was varied by connecting it through a multiposition switch to galvanometers of different dynamic characteristics or by partially closing a stopcock connection between the strain gage and indwelling arterial needle. The response of the manometer systems to sine and square wave pressures was recorded after each experi-

ment by use of a hydraulic oscillator (ISAACSON, JONES. *Am. J. Physiol.* 163: 722, 1950.) The brachial minus radial pressure differences in 17 normal subjects with use of radial and brachial manometers of similar dynamic response were: systolic,  $-6 \pm 0.9$ , diastolic,  $2 \pm 0.4$ , mean  $1 \pm 0.6$  mm. Hg. (The numbers following the  $\pm$  signs are the standard errors of the means.) Significant differences in the radial systolic pressure could not be detected in 11 normal and 5 hypertensive patients when manometer systems with uniform response out to 6 cps and above were used. However, a decrease in radial systolic pressure averaging 4 mm. Hg ( $P$  value  $< 0.01$ ) was detected in recordings from an overdamped manometer system with uniform response to 1 cps. Radial systolic pressures were not significantly different when recorded with underdamped and overdamped manometers with sensitivities at 40 cps of 290 and 35% of static response, respectively. None of the manometer systems produced significant variation in diastolic or mean radial minus brachial pressures. Distortion of the pulse contour as determined by alterations in the onset or build-up time of the pulse wave could be detected, at the chart speed used (20 mm/sec.), only in the recordings from the overdamped systems having a uniform sensitivity of 0 to 4 cps. Studies carried out in 8 subjects indicated that the dynamic response requirements for recording brachial pressure may be slightly less than for intraradial pressure.

*A direct reading cuvette oximeter and strain gage manometer assembly for measurement of blood oxygen saturation and continuous recording of arterial pressure and time-concentration curves of Evans blue dye in arterial blood of man.* EARL H. WOOD AND JOHN W. NICHOLSON, III\* (with the technical assistance of ROY ENGSTRÖM). Mayo Foundation, Univ. of Minnesota, and Mayo Clinic, Rochester, Minn.

The whole-blood cuvette oximeter (WOOD. *Medical Physics*, vol. II) has been converted to a single-scale device in which a circuit similar to that for the ear oximeter (*Proc. Staff Meet., Mayo Clin.* 25: 384) is used. Manometric and photometric determinations of arterial oxygen saturation were made in 30 subjects during breathing of air and 100% oxygen. The standard deviations of the values obtained by Van Slyke and cuvette analyses were 1.2 and 0.8% during oxygen breathing and 2.0 and 1.4% during air breathing, respectively. The standard deviations of differences between cuvette readings and Van Slyke analyses of samples of venous blood (average oxygen saturation: 75 (43-94)%) was 4.5%. The polythene cuvette tubing (2.8 mm. outside diameter; 2.0 mm. inside diameter) extends approximately 16 cm. on either end of the cuvette. One end terminates in a glass adapter for connection with indwelling arterial or venous needles or catheters. The opposite end terminates in a 2-way stopcock, one arm of which is connected to a strain

gage manometer (range  $\pm 760$  mm. Hg) for recording blood pressure while the other is used for intermittent sampling of arterial or venous blood (*Federation Proc.* 7: 137) or for withdrawal of a sample at measured rates of flow during continuous photographic recording of dye dilution curves (NICHOLSON, WOOD. *Am. J. Physiol.* 163: 738, 1950). The dynamic sensitivity of the over-all strain gage manometer cuvette system when attached to a 20-gage needle is uniform to 20 cps when a recording galvanometer (natural frequency: 40 cps) with the proper degree of electromagnetic damping is used. This dynamic response is adequate for most recordings of peripheral arterial pressure in man (WOOD. *Am. J. Physiol.* 163: 762, 1950).

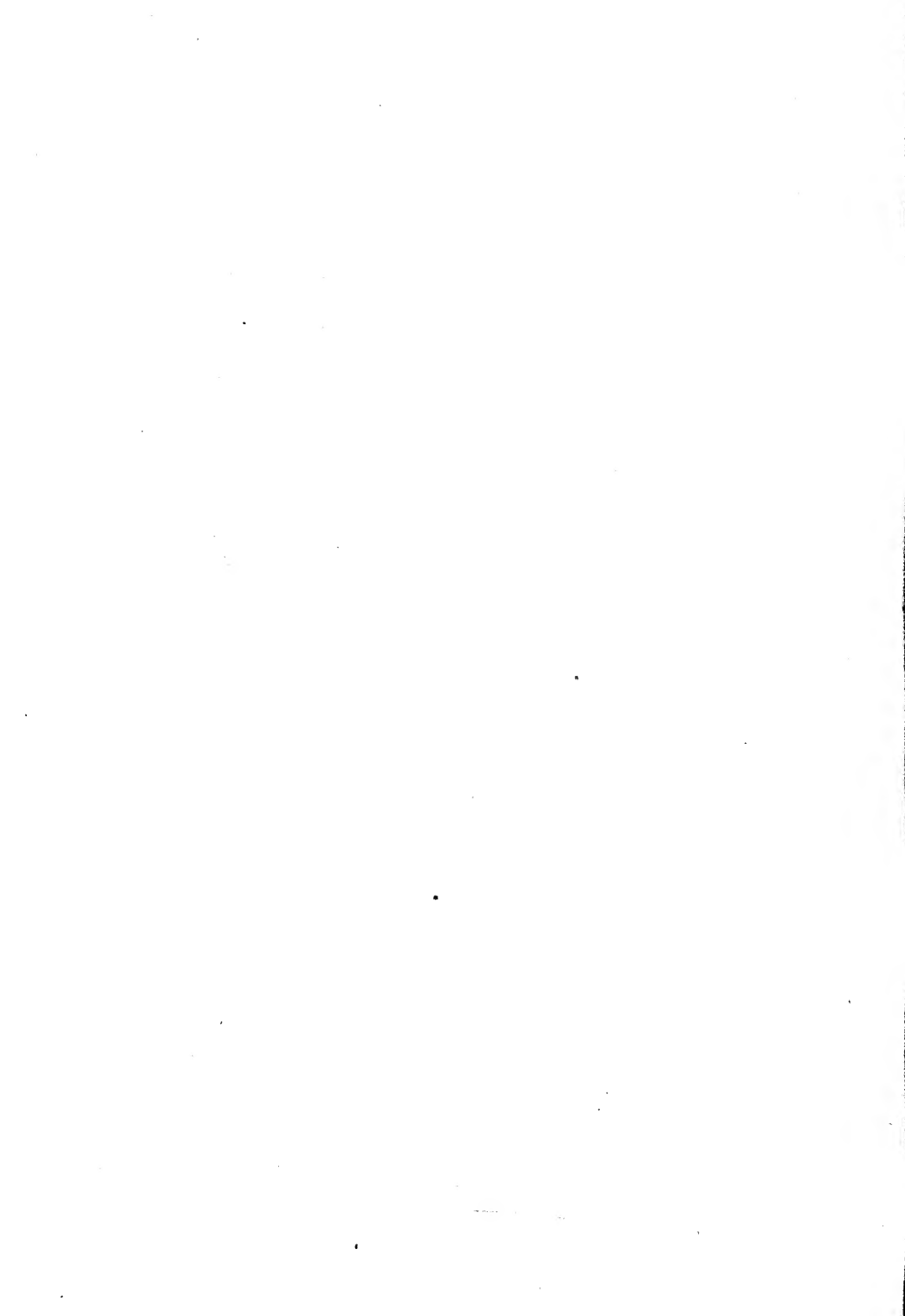
*Studies of intraperitoneal pressure as reflection of respiratory activity.* ALAN C. WOODS, JR. AND DONALD F. PROCTOR. Depts. of Surgery and Otolaryngology, Johns Hopkins Med. School, Baltimore, Md.

An attempt has been made in anesthetized dogs to learn the significance of the inspiratory phase of the pneumotachogram. This question has been explored by study of the pneumotachogram, intraperitoneal and intrapleural pressures. In addition cinefluorographic pictures synchronized with the pneumotachogram have been taken in the Department of Radiology at the University of Rochester. A negative dip in intraperitoneal pressure coinciding with the initial acceleration of inspiratory air flow has been observed. The positive rise in intraperitoneal pressure coincides with the maintenance of velocity of inspiratory air flow. The pressure falls to atmospheric (or in a negative direction) as expiration begins. Bilateral phrenicectomy in 3 dogs and spinal cord section at C-7 in 2 other dogs have indicated that the inspiratory phase of the pneumotachogram may represent the superimposition

of the early action of the intercostals and the later action of the diaphragm.

*Somatotopic organization of 'supplementary motor area' of the monkey.* C. N. WOOLSEY, P. H. SETTLAGE\*, D. R. MEYER\*, W. SENCER\*, T. PINTO HAMUY\* AND A. M. TRAVIS.\* Dept. of Physiology, Med. School, Univ. of Wisconsin, Madison.

Schaefer and Horsley (1888) and the Vogts (1919) described head and arm movements produced by stimulation of the medial aspect of the cerebral hemispheres of monkey rostral to the precentral leg area and dorsal to the sulcus cinguli. Little attention has been accorded these observations. Recently Penfield and Welch have described a 'supplementary motor area' in the same general location in man. On the hypothesis that the 'supplementary motor area' might constitute a second motor area analogous to the second somatic sensory area we have investigated the details of its organization and have found that the whole body is somatotopically represented in it. Face and arm portions of the area are in part on the free surface of the mesial aspect of the hemisphere but trunk and leg are represented almost entirely on the dorsal bank of the sulcus cinguli. The pattern is so disposed that lower face is nearest the rim of the hemisphere at the rostra level of the corpus callosum. The upper face, ear, neck and back, as far as tail, are represented in sequence along the deepest part of the sulcus cinguli. Fingers are represented at the edge of the hemisphere at the rostral limit of the precentral motor area, while more proximal parts of arm are activated from points between the finger area and the deeper parts of the sulcus cinguli. Leg points extend backward on the upper bank of the sulcus cinguli with proximal limb more rostral and digits more caudal.



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